PLAQUE MUTANTS OF WESTERN EQUINE ENCEPHALITIS VIRUS

by

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INTRODUCTION

The technique of plaque titration has enabled a quantitative assay method as well as the detection of certain genetic properties of viruses not otherwise recognizable. As a result of the ability to detect these properties the mapping of genetic markers or hereditary determinants of certain bacteriophage nucleic acids has become possible and such studies contribute to the basic understanding on the mechanism of life and heredity.

While preparing a stock culture of Western equine encephalitis (WEE) virus isolated from a single plaque of an infected monolayer culture of primary chick embryo cells, two strains of WEE virus distinguishable by differences in plaque size from each other and from the parent virus were isolated. Differences in plaque dimensions produced by serologically related strains of virus on a given susceptible monolayer cell culture are without doubt genetically controlled, but the precise physiological properties affected should be detectable through planned experimental techniques. Certain factors responsible for the differences in plaque morphology may be (1) rate of adsorption, (2) rate of virus synthesis or release, and perhaps (3) viscosity of the overlay medium.

This thesis problem was therefore undertaken to determine

what experimental evidence could be found that may account for the differences in plaque size between the two strains of WEE virus.

LITERATURE REVIEW

To obtain a basic understanding of WEE virus, a summary of certain properties and characteristics of this virus is initially presented. Since the nature of the problem presented in this thesis has been investigated by others using different viruses, certain pertinent and interesting points concerning studies with variants as reported in the literature are included.

I. PROPERTIES OF WESTERN EQUINE ENCEPHALITIS VIRUS.

A. Isolation of WEE Virus.

Though the encephalitic syndrome resulting from infection by WEE virus was observed for many years, the etiological agent was not isolated until 1931 by Karl Meyer, who was able to isolate the virus from the brain of an afflicted horse. Likewise, the first human isolate was made by Howitt (1939) from the CNS tissues of a child who died of this disease. It has since been shown that many animals and certain insects can support the propagation of the virus.

B. Transmission of WEE Virus.

WEE virus, classified within the Group A arthropod-borne viruses, is transmitted from animal to animal by means of a vector, which acts as a reservoir for the virus as well. For many years mosquitoes were suspected as the primary vector responsible for the transmission of WEE virus, but many factors had to be carefully considered before being generally accepted.

For example, Sulkin (1945) isolated WEE virus from chicken mites (Dermanyssus galinae) during an outbreak of the virus but could not induce any infection in normal chickens with the mites. Gwatkin (1939) isolated nymphs of Dermacentor andersoni fed on a viremic squirrel and recovered WEE virus, but there are no evidence to date indicating that ticks could reinfect other animals after several days of incubation.

Sudia (1959) showed that control chickens separated by a wire mesh in a mosquito proof enclosure from chickens infected with St. Louis encephalitis virus did not contract the disease. However, in another room under similar conditions but containing the mosquitoes Aedes aegypti, all control animals became infected. Donovan and Bowman (1942) could find no evidence that WEE virus was transmittable by direct contact in the human populace, but several accidental laboratory infections have been reported (Helwig, 1940; Gold, 1942).

Hammon et al. (1941) isolated WEE virus from Culex tarsalis during an epidemic. A few years later Hammon and Reeves (1943) demonstrated that Culex tarsalis could infect chickens from the tenth day after the mosquitoes were fed on viremic guinea pigs and ducks that were experimentally infected. Trager (1938) and later Chamberlain and Sudia (1957) found that mosquito tissues could indeed support the production of WEE virus and showed that there were an increase in titer of the virus from such tissues.

Of particular interest is the geographical restriction on the incidence to WEE virus infection, which is similar to many other viruses transmitted by mosquitoes. Generally, the incidence of WEE virus infection has been restricted to regions where mosquito species Culex tarsalis and Aedes aegypti were inhabited (Hammon et al., 1941).

C. Morphology and Chemical Constituents of WEE Virus.

WEE virus was shown by electron micrography to be a sphericle particle with a mean diameter of 40 to 55 mu (Sharp, et al., 1942). An unusual characteristic of WEE virus is the reportedly high lipoidal content of approximately 54% (Taylor, et al., 1943). The lipid may either be an integral part of the virus form of lipoprotein or firmly bound as a contaminant from the host tissue. Reporting on the lipid content of influenza virus Frommhagen et al. (1959) claimed that lipid analysis of viruses performed earlier by others were inaccurate and inconclusive. These investigators stated that discrepancy on total lipid from 20-40% found by other workers was basically traced to unsatisfactory methods of analysis.

Cheng (1958) has shown that trypsin, chymotrypsin, and papain inactivated the hemagglutinin and infectivity of Group B arthropod-borne viruses but did not affect the Group A viruses. Furthermore, Wecker (1959) was not able to extract infectious RNA from concentrated WEE virus by the standard cold phenol extraction procedure. He was, however, able to extract

infectious RNA when the extraction was performed at 50 C.

D. Infected Cells Observed in Electron Microscope.

Morgan et al. (1961) illustrated the appearance of WEE virus within infected cells as revealed in thin sections by the electron microscope. The earliest recognizable lesions were the formation of walled vacuoles with either single or double membranes. Precursor particles surrounding the vacuoles were also evident. Cytoplasmic granules were readily differentiated from the particles, which were "larger and averaging 22 mu in diameter, more uniform in size, more consistently spheriele with sharply defined margins, and more dense." These particles were not observable in thin sections of normal cells.

The membranes bordering the vacuoles appeared to be template sites at which the precursors were differentiated, and these sites occupied specific sides of the membrane. With single membraned vacuoles, precursor particles were formed outside the vacuoles, whereas, with double membraned vacuoles, synthesis occurred on both sides of the membrane but never between. Precursor particles forming outside the double membraned vacuoles appeared to cross the membrane and enter the lumen of the vacuoles, acquiring a peripheral coat in the meantime.

The vacuoles then migrated toward the cytoplasmic membrane. The illustrations suggested that the vacuole membrane formed part of the cell wall such that ejection of virus

resulted without loss of cellular integrity or dissolution of the cytoplasm. With doubled membraned vacuoles, the outer membrane formed the cell wall whereas the inner membrane protected the precursor particles within the lumen. Such particles, however, were believed to be unstable and disintegrated outside the host cell.

Frequently precursor particles appeared to have been detached from template sites and dispersed through the cytoplasm. Such particles migrated toward the cell wall and eventually moved outward, acquiring a protective coat and peripheral membrane. Extrusions of virus at the cellular surfaces have been observed with both WEE virus and influenza virus infected cells (Morgan et al, 1956). These investigators believed that through these processes which accompanied cellular loss that cytopathic effect was mediated.

E. Stability of WEE Virus.

Shahan and Eichhorn (1941) found that continuous propagation of WEE virus in 9 to 12 day old chick embryo for 226 successive days or an interruption through horse or guinea pig infection after 115 days did not alter the virulence for horses nor the original immunogenic properties for guinea pigs. Lyophilized Eastern equine encephalitis virus propagated in chick embryo and stored under refrigeration temperature remained virulent for only 3 months. Taylor et al. (1940) have demonstrated that the pH optimum for stability was between 7

and 8.5 for the Group A viruses.

WEE virus is considered to be relatively unstable. Lockart and Groman (1958) have determined the stability of WEE virus in different solutions at 37 C. The half-life of the virus was found to be approximately 4 3/4 hours when suspended in 40% horse serum, 40% Parker's medium 199, 17% Hanks balanced salt solution, and 3% of a 2.8% $NaHCO_3$. In a medium consisting of 80% Earles' saline, 10% chick embryo extract, and 10% horse serum, the half-life was 4 hours. However, in a protein defi-- cient medium as phosphate-buffered saline (PBS) the half-life was drastically reduced to 17 minutes.

When the experiment was repeated with PBS previously permitted to remain in contact with prewashed monolayer cultures for one hour, the inactivation of the virus was not as severe. At 60 minutes only 3 to 20% of the virus was inactivated in treated PBS while 85 to 95% reduction occurred in untreated PBS. Lockart and Groman (1958) suggested that possibly cysteine or other reducing substances were released from the cells into PBS during the contact period and that these substances were responsible for the significant slowing of WEE virus inacti- vation, an observation similarly noted with Eastern equine encephalitis virus at 37 C by Labzoffsky (1946).

F. Susceptibility of Cells to Infection by WEE Virus.

Nearly all investigations involving WEE virus pri- mary chick embryo and cells were used as the cell culture lines.

Recently, other cell lines capable of supporting WEE virus synthesis have been reported. Weinstein et al. (1956) found that human amnion cells undergo approximately a 50% cell degeneration when infected with WEE virus. Kissling (1957) and Rosenberger and Shaw (1961) have shown that complete cell generation was observed at 48 hours in infected hamster kidney cell cultures. Kissling (1957) also noted that guinea pig kidney cell culture was highly susceptible to infection of WEE virus, whereas dog kidney cell culture was completely resistant.

G. Plaque Morphology by WEE Virus.

Lockart and Groman (1958) have reported that chick embryo monolayer cell culture infected with WEE virus produced plaques measuring 4 to 5 mm in diameter. Their method of plaque titration followed the procedure of Dulbecco and Vogt (1954). Neutral red solution was added 48 hours later to the overlay medium, which consisted of 10% horse serum, 10% chick embryo extract, and 0.5% lactalbumin hydrolysate in Earles' balanced salt solution.

When monolayer cell cultures were seeded with an inoculum containing an overwhelming majority of 37 C inactivated virus, plaques measuring 1 mm in diameter were formed. These investigators have correlated the minute plaque formation to the phenomenon of homologous interference. It was found that the normal plaque sizes were attained with progressively larger dilutions. Furthermore, interfering activity was lost when in-

active virus preparations were diluted beyond an inactive virus cell ratio of one. It is an established phenomenon that cells of certain lines will not develop plaques when infected with specific viruses although the cells are highly susceptible to infection by these viruses and show complete cytopathogenicity under a fluid system. Presumably, the agar in the overlay medium has a direct influence since such cells appear normal when overlayed. For example, though L cells were capable of supporting growth of WEE virus with accompanying cytopathic effects, Chambers (1957) reported that plaques could not be seen under standard conditions.

However, Liebhaber and Takemoto (1961b) showed that with cells susceptible to infection by certain strains of coxsackie viruses but which failed to produce plaques, the addition of an anion exchanger, DEAE dextran, in the overlay medium enabled plaques to be formed.

H. Growth Curve of WEE Virus.

Susceptible cells infected with an animal virus first undergo a latent period then to an exponential rise period followed by a stationary phase of virus release. Dulbecco and Vogt (1954) were the first to report on the growth curves of WEE virus infected to monolayer and suspended chick embryo cell cultures. They found that in both cases a latent period of 2½ hours was observable with multiplicities of 4 and 3½ hours with a multiplicity of 0.15. Within 8 to 10 hours

maximum virus release per cell was attained. At 12 hours 200 plaque forming particles (PFP) were released per cell in suspended cell systems and approximately 1000 PFP per cell with monolayer systems.

When cells were suspended in Earles' saline solution virus synthesis did not take place. Possibility of cell deterioration was the explanation given by Dulbecco and Vogt (1954) since virus synthesis did occur when infected monolayer cells were incubated in Earles saline. Virus growth did not appear to be affected by pH fluctuation of 7 to 7.8 (Dulbecco and Vogt, 1954).

These investigators also found that initial cell infection must be initiated in a monolayer system, since cells in suspension adsorbed virus poorly. For example, at 50 minutes only 15% of the virus adsorbed to suspended cells whereas similar numbers of virus particles adsorbed to monolayer cells within 3.33 minutes. Adsorption period of 30 minutes was found to be adequate with 85 to 90% of the virus being adsorbed.

Rubin et al. (1955) reported on the extracellular and intracellular maturation of WEE virus in suspended chick embryo cells. Intracellular virus, which was not detectable up to the first hour, always remained between 4 to 10 per cell and were released approximately 0.9 minutes after attaining the property of infectiousness. The cells were disrupted by ultrasonic treatment, which did not appear to affect the in-

fectivity of the particle, and the titer of untreated and ultrasonic treated virus suspensions were similar.

Lockart and Groman (1958) repeated the experiments of Dulbecco and Vogt (1954) using monolayer systems with chick embryo and L cells. Infected L cells showed a longer lag phase in comparison to chick embryo cells and reached maximum virus release 28 hours later. The average yield per L cells was 200-700 plaque forming units (PFU) compared to 2500 PFU/cell with chick embryo cells.

I. Homologous Interference.

While propagating the PR8 strain of influenza A virus in chick embryo, von Magnus (1951) noticed that a higher titer of infective virus was obtained from the allantoic fluid if the inoculum contained less virus. Chick embryo infected with a 10^{-6} dilution of virus suspension yielded infective virus particle and hemagglutinin titers which were high and uniform. In serial transfer of undiluted allantoic fluid virus the infectivity titer decreased markedly and progressively through the first three transfers while the hemagglutination titers attained were maximal or nearly maximal. Von Magnus calculated that on the 3rd passage 1 out of 10,000 particles at the most were fully active. He thus suggested that the phenomenon could have been due to incomplete virus particles which represented an intermediate immature form of the fully active virus. Later Finter et al. (1955) found that the phenomenon was pro-

nounced when "serial seeds" were obtained under conditions permitting extensive inactivation of infectious virus during the individual passage. Correspondingly, the phenomenon was reduced but not abolished when precautions were taken to avoid accumulation of inactivated virus in the inoculum. Pauker and Henle (1955) showed that the yield of infectuous particles and hemagglutinin titers were nearly identical with an inoculum consisting of either heated virus pool (partially inactivated) or undiluted allantoic fluid virus.

Similar observations with WEE virus were made by Chambers (1957) on infected L cells. She noted an inverse relationship existing between size of viral inoculum and extent of cellular degeneration. When cultures of strain L cells were infected with low multiplicities of WEE virus, complete cell degeneration was observed within 56 hours. When much higher multiplicities were used, great proportions of cells continued to proliferate. These cells were not resistant mutants in that they were equally susceptible to challenge by a small inoculum of WEE virus after thorough washing and following a period of exposure to the virus antiserum. In cultures of L cells chronically infected with WEE virus, thermal inactivation of virus undoubtedly was a continuous process. Therefore, she reasoned that thermal inactivated virus may have served as the interferring agent that permitted the persistance of healthy multiplying cells despite the presence of

infectious particles.

As previously mentioned WEE virus was relatively unstable and when a huge amount of inactivated virus was plated on monolayers, minute plaques were readily seen (Lockart and Groman, 1958). Lockart (1960) further investigated the phenomenon and found that cells infected with 10 to 50 PFU/cell of WEE virus were completely destroyed within 48 hours. However, when cells were infected with 40 PFU each of both active and inactive virus per cell, slight cell degeneration with complete recovery a few days later were observed. The protection also was accompanied by a reduced viral yield. Cells exposed to inactivated virus for 24 hours followed by thorough washing were shown to be resistant to challenge with active virus from 2 to 5 generations of growth in the absence of any added inactive virus. The resistance was shown to be transient, however, as cells regained complete susceptibility after at least 5 generations of growth.

II. VARIATION OR MUTATION OF ANIMAL VIRUSES

A. Plaque Variants.

Variants of serologically homologous viruses, particularly in relation to plaque morphology, were noticed earlier with phage-bacterium systems. One observation noted was that phages adsorbing early produced much larger plaques than those adsorbing late (Sagik, 1954). On the other hand Doerman (1948) found that due to the phenomenon of "lysis in-

hibition" caused by superinfection with phage during the latent period, phage T2r$^+$ when plated on strain B Escherichia coli produced a small fuzzy plaque. In contrast T2r derived from T2r$^+$ by mutation, demonstrated a larger plaque with sharp outline.

Variants or mutants have also been observed with animal viruses. In order to study the biological properties of poliovirus by the plaque technique, Dulbecco and Vogt (1955) passed type 1 poliovirus (Brunhilde strain) through serial passages on monolayer cynomolgus monkey kidney cells. At the 15th passage different type plaques were observed, the wild type being distinguishable by the "mottled" type rims in contrast to the sharper rims of the mutants. The mutant strains also showed reduced pathogenicity by intracerebral inoculation in monkey. These investigators found that the mutants were more observable in overlay medium containing chick embryo extract, which is suboptimal for plaque development by poliovirus. In overlay medium containing monkey serum, which is optimal, the wild and mutant strains were undistinguishable. Therefore, the mutants where isolated in presence of embryo extract without monkey serum. Growth curve experiments between these two strains in monkey serum showed no differences in latent period, but the experiment repeated in chick embryo extract revealed longer latent period and slower rise during the exponential rise period by the wild type.

While developing the attenuated live poliovirus vaccine Sabin (1957) found a definite correlation between neurotropism and plaque size in vitro for the type 2 and 3 poliovirus but not for the type 1. He observed that the smallest plaques were produced by the most highly attenuated strains.

Dubes and Chapin (1956) obtained cold-adapted genetic variants of poliovirus strains Akron (type 1), Brooks (type 2), and Mabie (type 3) through passage at 30 C on monkey kidney cells. The 30 C passaged viruses demonstrated a more rapid cytopathogenic action on monkey kidney cells at 30 C in comparison to the original viruses. Furthermore, the cold-adapted variant particles produced larger plaques at 30 C than their progenitor particles. Cold-adapted Akron and Brooks strains showed no differences in virulence but Mabie strains did demonstrate virulence to a lesser degree.

Stanley et al. (1956) isolated a heat resistant strain of MEF (type 2) poliovirus. The activation curve of the resistant strain was significantly different than those obtained for the parent strain when exposed to 50 C heat for 40 minutes. To eliminate the possibility that aggregates of virus particles may be protecting some of the particles from heat, the fluid was passed through Seitz filter pad and centrifuged at 10,000 rpm for 1 hour. Without further exposure to heat there were no reversions to the inactivation curve even

after 4 passages through cell cultures.

Hearn and Soper (1961) studied the correlation of virulence and plaque size in Venezuelan equine encephalomyelitis (VEE) virus. The large plaque formers which measured 2 to 7 mm in diameter were lethal for mice by the intraperitoneal route, whereas the small plaque formers measuring 0.5 to 2 mm were nonlethal. Virus from the large plaques was found to produce about 6 to 10% small plaques consistently.

Fuerst (1961) isolated a number of plaque type mutants of murine encephalomyocarditis (EMC) virus. Size of the mutants were readily differentiatable by the plaque size and morphology.

B. Role of Bicarbonate in Detection of Mutants.

Vogt el al. (1957) have isolated type 1 poliovirus mutants with reduced pathogenicity for central nervous systems of monkeys that had a low plating efficiency at a low bicarbonate concentration of 0.11%. Titers obtained from infected monolayer cell cultures at acidic pH was a thousandfold to a millionfold less than under alkaline conditions. These mutants labeled d (delayed) exhibited dependency on bicarbonate concentration. Vogt et al. (1957) found that the delay in plaque formation was not due to inhibition of virus adsorption, multiplicities, or reduction in growth rate due to decreased susceptibility occurring within a period of 12 hours.

Hsiung and Melnick (1958) similarly studied the

effects of bicarbonate concentrations on plaque formation by virulent and attentuated strains of polioviruses. Sodium bicarbonate at 0.11% was used in the overlay medium as the lower concentration and 0.45% as the higher concentration. The plaque from virulent strains on monolayer cell cultures overlaid with the lower bicarbonate concentration were smaller than those overlaid with the higher concentration. Except for type 2 poliovirus, types 1 and 3 showed no significant differences in plaque counts at various bicarbonate concentrations. The attenuated strains on the other hand produced larger plaques at the higher bicarbonate concentration, but failed to produce any plaques at the low bicarbonate concentration. They have ultimately found that bicarbonate concentration rather than initial pH of the overlay exerted the effect on plaque development.

Hsiung and Melnick (1958) further noted that there were no differences in rate of adsorption and rate of virus multiplication for either virulent or attenuated strains at low bicarbonate concentration even when the inoculating dose of virus was large or small. These investigators concluded that mutation in the d direction was associated with attenuation which may have also resulted from other genetic changes in the virus. Horstmann et al. (1957) believed that among the attenuated polioviruses any back mutation to virulence proceeds in small steps.

C. Role of Inhibitors in Detection of Mutants.

Cells require serum or a serum factor to promote attachment to the glass surface. Earlier investigations involving certain adult bovine sera indicated that the presence of a non-specific inhibitor neutralized virus particles (Hammon et al., 1947; Bartell, 1955). Takemoto and Habel (1959) found that the inhibitor against type 1 poliovirus was associated with the gamma globulin of certain horse sera and that it differed from the serum inhibitor in not having the neutralizing ability. They could find no evidence that the inhibitor was a viral antibody, although the activity was specific for type 1 poliovirus and not for type 2 or 3 polioviruses, nor for coxsackie A9 and vaccinia viruses.

Casals and Olitsky (1947) have detected inhibitors in mice, hamsters, rabbits, and horse sera that were lipid in nature and which inhibited St. Louis, Russian Far East, and Japanese B encephalitis viruses. Similarly, Koprowski (1946) has shown that sera from rodents and marsupials contain an inhibitor which inactivates yellow fever, Japanese B, St. Louis, and West Nile encephalitis viruses. Results from the investigations on the pathogenisis of neurotropic viral infections indicated that these lipid inhibitors in serum probably played no great roles in resistance, since a stage of viremia occurred regularly and virus was readily isolated from blood. Ginsberg (1960) believes that these inhibitors were

chiefly viral receptor substances or other cell components which upon normal cell breakdown have been solubilized and escaped into the blood stream.

The addition of inhibitory horse sera (IHS) at concentrations of 2½% in overlay medium caused a marked reduction in plaque size with the wild type poliovirus type 1. Plaque counts were not affected at this concentration, but at 5%, a 50% reduction in count was noticed (Takemoto and Habel, 1959). Occassionally the occurrence of inhibitor-resistant strains capable of producing normal size plaques in the presence of IHS was noticed. When clonal isolations of the two strains were made and growth rate studies performed, differences in the growth rate was not detected. However, attachment and release time were found to differ.

Kanda and Melnick (1959) found that stable monkey kidney cells infected with the attenuated strains of poliovirus, which also formed small plaques, produced only 1 PFU/cell in comparison to 100 to 200 PFU/cell for the large plaque formers, or the virulent strains. Similarly, Sabin (1957) found that the attenuated type 2 and 3 poliovirus yielded only 1/32 to ½ as much virus per cell as the virulent parents.

Takemori et al. (1957) isolated small plaque mutants of poliovirus, and the isolation of such variants was facilitated by serial passages of the wild parent type in HeLa cells and in the presence of inhibitor containing normal bovine

serum (INBS). Upon further studies with these mutants Takemori et al. (1958) found that both strains showed a similar growth rate and a diminishing sensitivity to INBS with successive growth cycles. These mutants did not show any differences in kinetic curves of neutralization with antiserum nor changes in antigenicity and virulence. It was further shown that the neutralizing substances in the INBS were not antibodies but nonspecific serum inhibitors. These investigators hypothesized that such mutants may have a changed surface configuration which prevented effective combination with the NBS inhibitant without apparent effect on the "critical site" on the virus particle responsible for the combination with the antibody molecule, a phenomenon previously reported by Dulbecco et al. (1956).

Using normal bovine serum (NBS) Nomura and Takemori (1960) isolated by the limited dilution technique m (minute plaque) mutants of poliovirus with a mean diameter of less than 0.5 mm. They assumed that the serum was responsible for these selections, since the selection of m mutants was more rapid with 20-40% NBS and lactalbumin-yeast extract (LA-YE) medium than with a 2% calf serum LA-YE medium. They further found that host cells appeared to play a role, since m mutants were readily isolated from specific lines of FL cell cultures with both Mahoney and Saukett strains of polioviruses. These cell lines resisted cytopathic effects but allowed the synthesis

of the mutants to proceed. No differences in the growth curve between m and m^+ mutants were observed.

Takemori and Nomura (1960) investigated the reverse mutation of minute plaque mutants when they observed that various m lines of polioviruses gave rise to mutants forming large plaques similar to those of m^+ wild-type viruses. These back mutants were produced during the growth of m virus under ordinary experimental conditions in cell cultures and were readily detectable on plates seeded with low dilutions of m mutant stocks. Agar overlay extract was found to inhibit the growth of m mutants, and the m^+ revertants were selected by propagating m virus in cell culture in the presence of agar overlay extract. The proportions of m^+ reverse mutation in several m mutant stocks were measured and found to be 10^{-4} to 5.5×10^{-5}. The rate of mutation from m and m^+ was calculated by the method of Newcombe (1948) and was found to be approximately 3×10^{-7} for an MEF-1 m mutant. Reverse mutation was also observed in vesicular exanthema of swine virus (McClain et al., 1958; McClain and Hackett, 1959).

Takemoto and Liebhaber (1961a) characterized two plaque types of encephalomyocarditis virus. When parent EMC virus was grown in Earles strain L cells, 99% of the plaques were highly irregular in shape attaining a diameter of 1 mm at 4 days, where the other 1% had a sharply defined boundary with diameters ranging from 8-10 mm at 4 days. Both strains

where found to be serologically identical without exhibiting any differences in growth rate, thermal inactivation rate, or virus yields per cell under ordinary conditions. In addition, both strains were equally virulent for adult mice inoculated either intracerebrally or intraperitoneally. The large plaque mutant, however, demonstrated a significantly higher hemagglutinating-PFU ratio.

Growth curve studies conducted with the two strains of EMC virus revealed that newly synthesized virus from r mutant (large plaque) infected L cells were not detectable in the supernatant 5 to 6 hours after infection, whereas, in the case of r^+ (small plaque, wild type) infected L cells, extracellular virus production was demonstrable as early as 3 hours after infection (Takemoto and Liebhaber, 1961b). These investigators confirmed the findings of Takemori and Nomura (1960) whereby plaque size suppression was found to be directly influenced by an inhibitor found in agar used in the overlay medium. When the overlay medium was frozen at -40 C and later thawed an extract was obtained. Takemori and Nomura (1960) found that the extract contained an inhibitor which affected the growth rate of virus synthesis by L cells infected with the r^+ mutant.

Araki (1959) reported that an extract obtained from a thawed agar solution previously frozen at -40 C contained a sulfated polysaccharide. He also identified D-

galactose and 3, 6-anhydro-L-galactose as the major monosaccharide in agar although the position of the monoesterified sulfate has not been definitely established.

Liebhaber and Takemoto (1961b) recently reported that the addition of DEAE detran, an anion exchanger, in the agar overlay medium at a concentration as low as 50 ug/ml enabled plaques produced by $\underline{r}^+$ to increase to the larger plaque size type. Takemori and Nomura (1961) obtained data which suggested that agar extract did not act directly on the poliovirus mutants to cause inactivation by combination or by a direct virucidal effect, but that it inhibited viral multiplication by altering primarily the metabolism of susceptible host cells.

D. Variation in Cytopathic Manifestation.

Variation in cytopathic manifestation has been observed in HeLa cells infected with herpes simplex virus. Under microscopic observations Ross and Orlans (1958) noted clumps of rounded cells as well as large multinucleated giant cells. Hoggan and Roizman (1959) later isolated two distinct strains of herpes virus by the limiting dilution technique that exhibited the cytopathic characteristics. One strain developed microplaques, which were small, pocklike and induced multilayered clumps of single rounded cells. Infection of HeLa and FL cells by the second strain was followed by formation of large multinucleated giant cells within macroplaques which attained

a diameter of 4 to 5 mm in 4 days. Both strains were equally neutralized by antisera against NT, Zachardie, Tucker, and HF strains of herpes virus. However, in the presence of antiserum cultures infected with microplaque variants usually survived, whereas, cultures infected with macroplaque variants invariably degenerated even though antibody present in the medium was sufficient to neutralize more than 99.99% of virus formed by infected cells. Hoggan et al. (1961) found that discrete plaques formed by the macroplaque variant were surrounded by giant cells produced in replicating FL Cell cultures if 2.5 to 20% human or 10 to 20% horse serum was present in the liquid medium. No plaques were observed if the medium contained 2.5 to 20% chick serum or less than 10% horse serum. However, plaques were produced in these "deficient" medium if 0.2% immune gamma globulin was added.

E. Variation of Cells to Virus Susceptibility.

Vogt and Dulbecco (1958) studied the properties of a strain of HeLa cells with increased resistance to poliomyelitis virus. A culture of HeLa S3 cells was exposed to poliovirus and later treated with poliovirus antiserum. From the surviving cells a HeLa cell culture was isolated which showed definite signs of resistance to poliovirus infection. The resistant cells, however, were found to adsorb virus as well as the sensitive parent culture.

Darnell and Sawyer (1959) similarly studied varia-

tion in plaque forming ability of HeLa cells to poliovirus infection. However, these investigators obtained their cell strains by clonal isolation and not by selection after virus exposure. HeLa cell line I-3 was found to adsorb virus at a more rapid rate than the less susceptible cell line S3-1. A difference in the appearance of the plaques formed by the different cell strains was also observed. The cell line I-3 produced plaques averaging 1.5 to 2 mm in diameter in contrast to 5 mm for I-3 cell lines. Therefore, there appeared to be no necessary relation between susceptibility or rate of adsorption and the size of plaque formed.

F. Studies of Attenuated Viruses.

Melnick (1951) isolated two immunologically related strains of poliovirus that showed differences in the degree of infectiousness following oral administration to monkeys. Twenty-one monkeys fed an attenuated strain of poliovirus failed to develop any antibodies, but a second group of monkeys developed paralysis and antibodies when fed the Yale-SK strain (virulent strain of poliovirus). When monkeys in the former group were later fed the Yale-SK strain, all animals developed antibodies. Monkeys inoculated intramuscularly with the attenuated strain did not develop any disease although they were left with antibodies capable of neutralizing the virus. It was subsequently found that the attenuated virus was no longer infectious for monkeys through the oral route.

Ledinko et al. (1951) infected cynomolgus monkey testis cell cultures with the identical strains mentioned above and found that 4 days after infection with the Yale-SK strain, complete degeneration of cells occurred in comparison to slight tissue destruction by the attenuated strain. These investigators believed that virulence was lowered significantly for the attenuated strain due to the numerous mouse passages previously undergone.

Enders et al. (1952) obtained variants of Brunhilde strain of poliovirus following cultivation in various in vitro systems. Variants passaged through cell cultures of human embryo skin muscle were especially less virulent for monkeys.

Li and Hable (1951) adapted a type 3 Leon strain of poliovirus to mice. The virus was highly virulent for mice and could infect mice only through intraspinal inoculation. After 72 mouse passages the virus was passed once through monkey testis cell culture. Of 8 mice inoculated with a titer of 10^{-4} only 2 mice became paralyzed. The virus lost all virulence for mice after a second passage through monkey testis cell culture. Similarly, pathogenicity for monkeys through the intraspinal inoculation was also lost. When monkey kidney cell cultures were infected with virulent and avirulent strains, the virulent strain infected cells degenerated after 24 hours in comparison to 120 hours with the avirulent strain.

Li et al. (1955) investigated the affects of testicular

cell culture passage with a type 1 poliovirus, which was able to infect mice via intraspinal route. Two passages of the virus through monkey testicular cell culture failed to alter its infectivity for mice through the intraspinal route, but the virus lost its infectivity for monkeys via the intracerebral route. Two chimpanzees which were given the virus intracutaneously did not develop any signs of infection, viremia, nor did they shed virus in their stool.

Roca-Garcia and Jervis (1955) experimentally produced an adapted strain of poliovirus type 2 to chick embryo. The virus did not show signs of pathogenicity for monkeys inoculated either through intracerebral or intraspinal inoculation. When monkeys were inoculated intravenously with 450,000 mouse LD_{50} doses, none of the animals became ill during the observation period nor showed evidence of inflammatory changes or appreciable cellular loss of the spinal cords. Similar results were obtained when monkeys were inoculated intramuscularly or orally. Furthermore, virus could not be isolated from stool samples. When the egg adapted virus was passed through monkey kidney cell cultures, the virus was invariably lost between the 18th and 23rd passages, although some cytopathogenic activity was apparent while the virus was recoverable. The virus propagated in monkey kidney cells retained its attenuated properties for animals with "no apparent tendency to regain virulence by serial intracerebral or intraspinal passages in monkeys."

MATERIAL AND METHODS

I. SOLUTIONS AND MEDIA.

A. Growth Media.

The growth medium used for the cultivation of primary chick embryo cells was composed of Earles' balanced salt solution, 0.4% lactalbumin hydrolysate, 3% calf serum, 0.5% of a 4.5% $NaHCO_3$ solution, and the antibiotics penicillin and streptomycin (100 units/ml and 100 ug/ml respectively). The culture media for the other cell lines used in the experiments contained 0.5% lactalbumin hydrolysate and increased concentrations of calf serum; 5% for mouse embryo, hamster, and guinea pig kidney cell cultures and 10% for the stable cell lines L, HeLa, and FL. All media were sterilized by positive pressure filtration using Seitz filters.

1. Earles' balanced salt solution was prepared in 10X concentrations and stored unsterilized at 4 C for only short periods of time. The formula of the solution used in the growth medium is as follows:

Phenol red	0.02 g/liter
NaCl	6.8 g/liter
KCL	0.4 g/liter
$NaH_2PO_4 \cdot H_2O$	0.14 g/liter
$MgSO_4 \cdot 7H_2O$	0.2 g/liter
$CaCl_2$	0.2 g/liter
Glucose	1 g/liter

The solution used in the growth medium for cultivation of stable cells was modified by increasing the glucose concentration to 3 g/liter.

2. Calf serum was obtained from a local slaughter house, and the serum separated and filtered through Seitz filters prior to incubation at 56 C for 30 minutes. The serum was stored in 200 ml volumes at -20 C until ready for use.

3. Streptomycin sulfate and crystalline potassium penicillin G were diluted with physiological saline, and unused portions of the antibiotics were stored at -20 C.

B. Phosphate Buffered Saline.

Phosphate buffered saline solutions were prepared according to the formula of Dulbecco and Vogt (1954) as follows:

NaCl	8 g/liter
KCL	0.2 g/liter
Na_2HPO_4	0.15 g/liter
KH_2PO_4	0.2 g/liter
$MgCl_2 \cdot 6H_2O$	0.1 g/liter
Anhydrous $CaCl_2$	0.1 g/liter

Solution of phosphate buffered saline deficient in Mg^{++} and Ca^{++} (PD) contained the other salts in similar quantities. PD and PBS were made in 10X concentrations and stored at room temperature for a short period of time. Both solutions were sterilized by Seitz filtration.

C. Trypsin Solution.

Trypsin solutions were made as described by Youngner (1954) with one modification. Difco (1:250) trypsin was dissolved in PD and the solution sterilized by filtration. Only trypsin solutions prepared within 48 hours and stored at 4 C were used.

D. Neutral Red Solution.

Neutral red was dissolved in distilled water (1:5000) and sterilized in the autoclave.

E. Purification of Agar.

Difco agar was purified according to the method of Dulbecco and Vogt (1954) with one modification. After washing the agar with acetone, two additional washings were made with ether.

F. Agar Overlay Media.

The washed agar was added to distilled water, sterilized in the autoclave, and allowed to equilibrate at 42.5 C in a temperature controlled water bath. Agar overlay media were prepared by mixing equal volumes of the 1.8% agar solution with twice concentrated growth media. The growth media used were similar to those described for cultivation of the various cell cultures.

II. CELL CULTURE TECHNIQUES.

A. Chick Embryo Cells.

The method used was that described by Welsh et al.

(1958) with modifications. Ten-day old chick embryos of the Hi-line strain were placed in a sterile dish containing a small volume of growth medium. After removing and discarding the heads and limbs from each embryo, the tissues were minced with a sterile scissors, transferred to a trypsinizing flask, and washed twice with small volumes of 37 C PD solutions. The washed tissues were trypsinized four times for three minute intervals using a magnetic stirrer.

The cell suspensions were filtered through sterile gauze into a flask containing 25 ml of calf serum and were kept at ice bath temperature until the entire extraction procedure was completed. Cells were collected by centrifugation for five minutes at 600 rpm in an International centrifuge with a size 1 SB head, resuspended in growth medium, and filtered again into a 100 ml graduated cylinder. The cell concentration was determined with the aid of a clinical hemocytometer before diluting the cell suspension with growth medium to a final concentration of 2×10^6 cells/ml.

The diluted cell suspensions were added to either screw cap culture tubes, 60 mm diameter Petri or Falcon plastic dishes*, or 16 oz prescription bottles depending on the nature of the experiment. The volumes dispersed were 1.5, 5, and 50 ml respectively.

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* Falcon Plastics, Los Angeles, California.

Cell cultures cultivated in Petri dishes were incubated in a 5% CO_2 atmosphere under conditions described by Bubel et al. (1956), but bottles and culture tubes containing the cell suspensions were sealed tightly with rubber stoppers or screw caps. All cell cultures were incubated for 48 hours at 37 C prior to use.

B. Mouse Embryo Cells.

Mouse embryos were removed from approximately fourteen-day old pregnant mice and the cell cultures prepared as described for chick embryo cells.

C. Guinea Pig and Hamster Kidney Cells.

The kidneys were removed from anesthesized animals, and placed in sterile Petri plates. The cortical tissues used for the cell cultures were then dissected free from the medulla with the aid of a scalpel. The remaining procedures were similarly performed as described for the chick embryo cell cultures except that the trypsinization time was increased to 10 minutes and the suspended cells were diluted to a final concentration of 5×10^6 cells/ml. The incubation time was also increased to four days or until confluent monolayers were present.

D. Stable Cells.

Stable cell lines HeLa, L, and FL, obtained from Microbiological Associates, Albany, California, were grown and maintained in 16 oz prescription bottles. To prepare cell

suspensions for transfer, the monolayer cell cultures were first washed with small volumes of PD solution, trypsinized with 1 ml of a 0.05% trypsin solution, and resuspended in growth medium at a concentration of 1 X 10^6 cells/ml. The incubation conditions were similar as described for the kidney cell cultures.

III. VIRUS ASSAY.

Virus was titrated by the plaque assay technique as described by Dulbecco and Vogt (1954) with few modifications. Two tenths ml of virus suspension prepared in growth medium was added to each cell monolayer previously washed twice with growth medium. Attachment was allowed to progress at 25 C for 1 hour before adding agar overlay medium to each monolayer culture.

When plaque morphology was the determinant factor, 1.5 ml neutral red solution was added to each plate 48 hours later and plaques observed after an additional 6 hours of incubation in 5% CO_2 atmosphere as described (Bubel et al., 1956). For quantitative analysis of virus concentration neutral red was added 24 hours later to overlayed monolayers infected with the LP-7 virus and 36 hours for monolayers infected with the SP-6 virus.

IV. VIRUS SOURCE.

The original parent virus was isolated from infected human tissue homogenate by inoculation of one-half-day-old

"wet" chicks. The virus was subsequently passed several times through mice by intracerebral inoculation prior to infecting garter snake embryo cell cultures maintained at 25 C. These cells developed extensive cytopathic changes. Fluid from the infected snake embryo cell cultures were then used as a source of virus to infect primary chick embryo cells. After the tenth passage a plaque measuring 5 mm in diameter and labeled as W-4 was selected and the virus progeny isolated, recloned, and used for further investigations.

RESULTS

I. ISOLATION OF PLAQUE MUTANTS OF WEE VIRUS.

During the course of early studies it was noted that upon several passages of WEE virus strain W-4 on monolayer cultures of chick embryo cells, plaques exhibiting different sizes and morphologies were noted. Fig. 1 illustrates three distinct plaque types produced by the W-4 strain of WEE virus. The diameters of the plaques were 2 mm for the smallest, 5 mm for the medium, and 8 mm for the largest.

When progeny viruses of a number of the smaller and larger plaques were isolated, passed through chick embryo cells, and titrated on monolayer cell cultures, in most cases plaques of various sizes were noted. However, one isolate, designated as SP-6, produced plaques with an average diameter of 2mm, and another, designated as LP-7, produced plaques 8 mm in diameter. Fig. 2 shows the plaques characteristic of SP-6 viruses and Fig. 3 of LP-7 viruses. The photographs also illustrated that the margins of LP-7 plaques were smooth and "entire", whereas the SP-6 plaques were irregular.

Strains 85, 2290, 1893, 1135, and 1392 of WEE virus was obtained from Dr. C. W. Ecklund at the Rocky Mountain Laboratory, Hamilton, Montana. Two of the strains of WEE virus originally isolated from mosquitoes were also titrated. Strain 85 produced plaques similar in diameter to those formed by SP-6 virus (Fig. 4). Strain 2293 on the other hand produced differ-

FIG. 1. Plaques of WEE virus strain W-4 on primary chick embryo monolayer cell cultures after 56 hours of incubation.

FIG. 2. Plaques of WEE virus mutant SP-6 on primary chick embryo monolayer cell cultures after 54 hours of incubation.

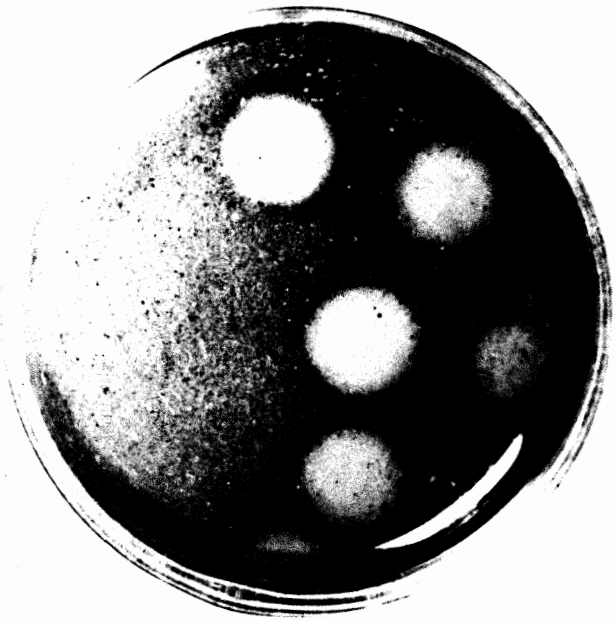

FIG. 3. Plaques of WEE virus mutant LP-7 on primary chick embryo monolayer cell cultures after 54 hours of incubation.

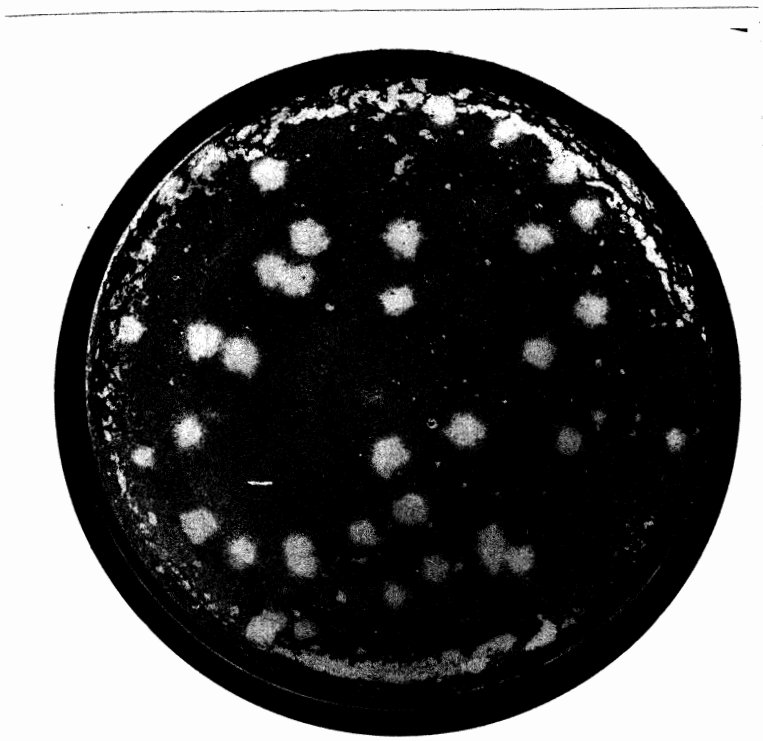

FIG. 4. Plaques of WEE virus strain 85 on primary chick embryo monolayer cell cultures after 56 hours of incubation.

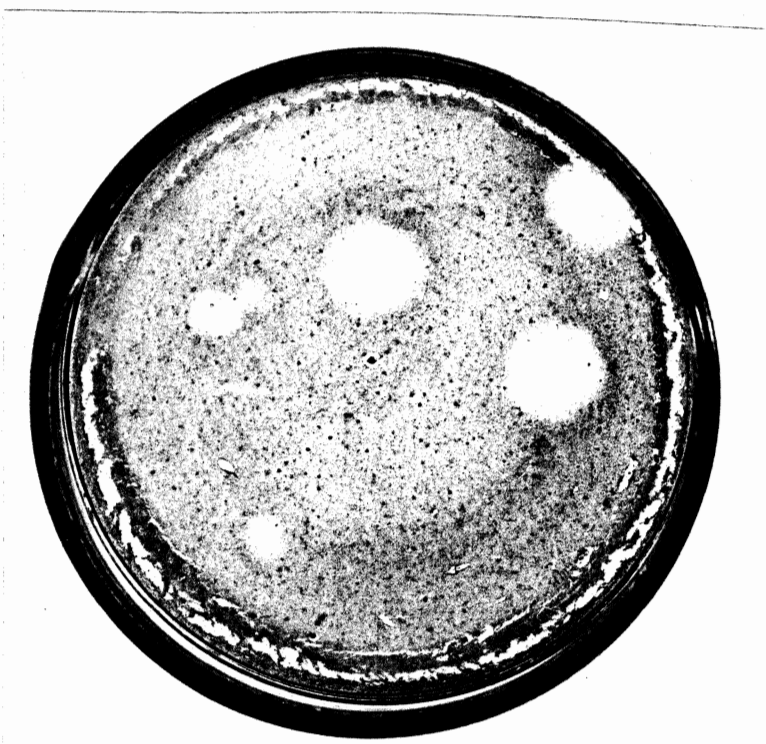

FIG. 5. Plaques of WEE virus strain 2293 on primary chick embryo monolayer cell cultures after 56 hours of incubation.

ent sized plaques most of which were 5 mm in diameter and a few 2 mm (Fig. 5).

II. SEROLOGICAL CHARACTERIZATION OF PLAQUE MUTANTS.

To determine whether both plaque mutants of WEE virus were antigenically homologous, one month old chicks were inoculated subcutaneously with 0.2 ml of inoculum containing 1 X 10^3 plaque forming units (PFU)/ml of either virus. The chicks were injected three weeks later with an inoculum containing 2 X 10^7 PFU. Sera obtained from the chicks after an additional 30 days were inactivated for 30 minutes at 56 C and used for neutralization experiments.

To determine the neutralizing antibody titers of the chick sera 0.5 ml of virus suspension containing 100 PFU was added to each tube containing an equal volume of antiserum diluted serially in growth medium. After 30 minutes of incubation at room temperature 0.5 ml from each virus-serum mixture was added to cultures of chick embryo cells grown in screw cap tubes. One hour later 2.5 ml volumes of growth medium were added. After 24 hours of incubation at 37 C, the screw caps were loosened and the cell culture incubated for an additional day. Cell degeneration as determined by microscopic examination was used as the criterion for the absence of neutralizing antibody.

Results of the experiments on neutralization tests using standard WEE virus antiserum obtained from Communicable

Disease Center (CDC), Atlanta, Georgia, and antisera prepared against each mutant are recorded in Table 1. The data indicated that both strains were specifically neutralized by the standard WEE virus antisera from CDC. Cross neutralization was also observed with antisera prepared against each strain, indicating that both SP-6 and LP-7 viruses were antigenically homogeneous with relation to neutralizing antibodies.

III. ANIMAL PASSAGE.

As further evidence to establish that the differences in plaque morphology were stable genetic traits, the effect of animal passage was investigated. Twenty-five-day old mice were injected intracerebrally with 0.025 ml of an inoculum containing approximately 10^3 PFU/ml of either SP-6 or LP-7 viruses. After 48 hours when neurologic disturbances were observed, the mice were sacrificed and the brain removed and homogenized in a sterile mortar. A 10% suspension of the brain homogenate prepared in growth medium was centrifuged for 5 minutes to remove cellular debris. The supernatant from each mice passage was diluted in growth medium and each dilution was titrated on monolayer cultures of chick embryo cells.

The results of these experiments are recorded in Table 2. It appeared that up to three mice passages by intracerebral inoculation failed to result in plaques with diameters other than those characteristic of SP-6 and LP-7 viruses.

TABLE 1

Determination of antigenic relationship between SP-6 and LP-7 strains of WEE virus.

Serum Source	Virus	Serum Dilution (Two Fold) 4	8	16	32	64	128	256	512
CDC Antiserum	SP-6	-*	-	-	-	-	-	-	+
CDC Antiserum	LP-7	-	-	-	-	-	-	-	+
SP-6 Antiserum	SP-6	-	-	-	-	+	+	+	+
SP-6 Antiserum	LP-7	-	-	-	-	+	+	+	+
LP-7 Antiserum	SP-6	-	-	-	-	-	-	-	+
LP-7 Antiserum	LP-7	-	-	-	-	-	-	-	+
Normal Chick Serum	SP-6	+	+	+	+	+	+	+	+
Normal Chick Serum	LP-7	+	+	+	+	+	+	+	+

* Assay based on microscopic observations for cell degeneration at 48 hours. (-) cells normal; (+) cells degenerated.

IV. FACTORS AFFECTING PLAQUE MORPHOLOGY.

Among those factors which have been reported to influence the development of plaques by certain animal viruses on monolayer cell cultures were composition of the overlay medium (Dulbecco and Vogt, 1955; Nomura and Takemori, 1960; Liebhaber and Takemoto, 1961a) and homologous interference (Lockart and Groman, 1958). A hypothesis was thus made that either a constituent of the agar overlay medium or homologous interference was affecting the development of plaques by the mutant SP-6 and LP-7 viruses. Therefore, experiments were done to determine if any of these factors was responsible for the observed differences in plaque sizes.

Purification of the agar according to the method previously described may have removed certain factors necessary for SP-6 plaques to increase in size. Therefore, agar overlay media were prepared using purified and unpurified agar and added to monolayer cultures of chick embryo cells infected with either SP-6 or LP-7 viruses. The data, as shown in Table 3, indicated that the purity of the agar used in the overlay medium had no observable effect on the formation of plaques by either mutant after 54 hours of incubation.

To determine whether plaque formation by the mutants of WEE virus was affected by inhibitory or nutritional factors present in serum, the effects of horse and calf serum in different concentrations in the overlay medium were investigated.

TABLE 2

Effect of intracerebral mice passages of SP-6 and LP-7 mutants of WEE virus on plaque morphology.

Passage Number	Virus Strain	Plaque diameter in mm at 56 hrs
1st	SP-6	2
	LP-7	8
2nd	SP-6	2
	LP-7	8
3rd	SP-6	2
	LP-7	8

TABLE 3

Effect of purified and unpurified agar on plaque morphology.

	Virus	Plaque Diameter in mm at 56 hrs
Purified agar:	SP-6	2
	LP-7	8
Unpurified agar:	SP-6	2
	LP-7	8

Agar overlay media were prepared to contain inactivated calf or horse serum in concentrations of 1.5, 3, 5, and 10%. It should be noted that the horse serum pool in these studies were examined for the presence of specific neutralizing antibody and were found to be lacking in neutralizing activity. Table 4 summarizes the observed effects of different concentrations of the calf and horse serum on the size of plaques produced by SP-6 and LP-7 viruses on monolayer cultures of primary chick embryo cells. Overlay media containing calf serum in the different concentrations did not affect the plaque sizes characteristic of the two mutants. However, the plaque diameters were considerably less in overlay media containing horse serum in any of the concentrations used.

There were instances in which the use of certain lots of calf sera resulted in an suppression in the size of plaques produced by both mutant strains, considerably more than even horse serum. However, such sera exhibiting these plaque suppressing properties were not investigated further.

Subsequent to the completion of these researches Liebhaber and Takemoto (1961a) earlier reported that the addition of diethylaminoethyl (DEAE) dextran to the agar overlay medium permitted small plaques of mutants of encephalomyocarditis (EMC) virus to produce plaques of the same size as those formed by the wild-type parent virus. Therefore, experiments were made to determine if an inhibitor in agar was similarly

TABLE 4

Effect of different concentrations of calf and horse sera in overlay medium on plaque morphology.

Serum Source	% Conc.	Diameter in mm at 56 hours SP-6	LP-7
Calf	1.5	2	8
Calf	3.0	2	8
Calf	5.0	2	8
Calf	10.0	2	8
Horse	1.5	1	4
Horse	3.0	1	4
Horse	5.0	1	4
Horse	10.0	1	4

suppressing plaque development by SP-6 virus.

The anion exchanger DEAE-Sephadex A-50, obtained from Pharmacia, Uppsala, Sweden, was used, because DEAE dextran was not commercially available. Since the preparation of the DEAE dextran-agar overlay medium was described by Liebhaber and Takemoto (1961b) in a later publication, various experimental procedures were utilized to determine the effects of DEAE-Sephadex on plaque development by the two mutants of WEE virus.

In each of the experiments a final concentration of 50 or 200 ug/ml of the anion exchanger was used in the overlay medium. These media were added to monolayers of primary chick embryo cells infected with the SP-6 and LP-7 strains of WEE virus. The results of the experiments are recorded in Table 5.

When the agar overlay media were prepared from agar and different concentrations of DEAE-Sephadex previously sterilized together and subsequently added to infected monolayer cell cultures, SP-6 plaques developed were 5 mm in diameter but LP-7 plaques showed no changes. However, DEAE-Sephadex in a final concentration of 200 ug/ml in the agar overlay medium caused the agar to lose the characteristic solidifying properties throughout the period of incubation, whereas, 50 ug/ml allowed the agar overlay medium to solidify.

The procedure described by Liebhaber and Takemoto (1961b) was then followed. The anion exchanger as commercially obtained was added to 42.5 C sterilized agar, and after incu-

TABLE 5

Effect of DEAE-Sephadex added to agar overlay media on plaques morphology.

Conditions of Preparations	Final Conc. DEAE-Sephadex	Virus Strain	Ave plaque diameter in mm at 56 hrs
Agar + DEAE-Sephadex (Autoclaved)	50 ug/ml	SP-6	5
		LP-7	8
Agar + DEAE-Sephadex	200 ug/ml	SP-6	5
		LP-7	8
Sterile Agar + DEAE-Sephadex (Unautoclaved)	200 ug/ml	SP-6	3-5
		LP-7	8
Control*		SP-6	2
		LP-7	8

* DEAE-Sephadex not added.

bation for 30 minutes, growth medium was added to the agar. The LP-7 plaques developed under the described agar overlay medium containing 200 ug/ml of DEAE-Sephadex were not affected but SP-6 virus produced plaques of mixed diameters ranging from 3 to 5 mm.

In a final procedure DEAE-Sephadex and agar were sterilized separately and the agar overlay medium subsequently prepared. When the infected monolayer cell cultures were overlayed with the described medium SP-6 and LP-7 plaques produced were undistinguishable from the control plaques.

The results from the previous experiment suggested that plaque development by SP-6 virus was affected by an inhibitor in agar. Therefore, the affect of overlay medium prepared with methylcellulose on plaque formation was investigated.

DOW "Methocel" of grades 100 cps and 4000 cps were slowly added to 95 C distilled water as prescribed by the directions to "wet" the powders. However, temperatures of the sterile and unsterile preparations were not allowed to go below 42 C, but were mixed with 42 C growth medium and added to infected monolayer cell cultures. The culture plates were maintained on a cool, moist towel for a few minutes until the methylcellulose dissolved completely. The monolayer cell cultures were incubated at 37 C in a closed CO_2 atmosphere chamber as previously described.

Since the overlay media did not solidify throughout

the period of incubation, 1.5 ml of neutral red solution was added carefully a drop at a time over the entire surface of the overlay media 48 hours later. The neutral red was diffused completely throughout the overlay medium within a few minutes. The experimental results are shown in Table 6.

The overlay media prepared from autoclaved "Methocel" of grades 100 and 4000 cps at 1 and 2% final concentrations suppressed the development of SP-6 and LP-7 plaques by 50%, but the differences in plaque diameters were still evident. Overlay media containing unsterile "Methocel" 4000 cps affected plaque formation by both viruses. The SP-6 plaques were barely visible with counts being less than the calculated titer of the virus suspension, and LP-7 plaques averaged 1 mm in diameter.

One of the hypothesis made earlier was that homologous interference was responsible for the development of SP-6 plaques. To test this hypothesis, suspensions of SP-6 virus particles inactivated in 37 C water bath for a period of 5 days were added to monolayer cultures of primary chick embryo cells at a multiplicity of 2. After incubation for 12 hours at 37 C the monolayers were washed and infected with a low titer of active SP-6 and LP-7 viruses. At 56 hours the plaques formed by LP virus were 4 to 5 mm in diameter and those formed by SP-6 virus were 0.5 to 1 mm (Table 7). These results of plaque suppression due to homologous interference

TABLE 6

Effect of methylcellulose substituted for agar in the overlay medium on plaque morphology.

	% Conc.	Plaque Diameter in mm at 56 hours SP-6	LP-7
Methylcellulose 100 CPS			
Sterilized	1%	1	4
	2%	1	4
Unsterilized	1%	*	*
	2%	*	*
Methylcellulose 4000 CPS			
Sterilized	1%	1	4
	2%	1	4
Unsterilized	1%	0-0.5	1
	2%	0-0.5	1
Control		2	8

* Not done.

TABLE 7

Monolayer cells exposed to inactivated SP-6 WEE virus for 12 hours prior to infection with active virus.

Active Virus	Diameter plaques in mm at 56 hours
SP-6	0.5 to 1
LP-7	4 to 5
SP-6*	2
LP-7*	8

* Control plates not exposed to inactivated virus.

confirmed the findings of Lockart and Groman (1958).

A suspension containing SP-6 virus was diluted 4 logs beyond the dilution giving 5 plaques per plate. Each dilution was left overnight on separate monolayer cell cultures. After washing with growth medium the monolayers were challenged with a low concentration of active virus of both mutant virus. Such plates revealed plaque sizes characteristic of each virus mutant, thereby indicating that inactive virus particles were absent in the original diluted suspensions.

Cells from primary chick embryo fibroblasts grown in culture tubes were each infected with SP-6 and LP-7 viruses. After 1 hour, the cells were washed 5 times with growth medium and allowed to incubate at 37 C for 4 hours. The monolayers, in this period of active virus synthesis, were again washed with warm growth medium 5 times. After an additional hour of incubation the fluids containing virus particles no more than an hour old were titrated. Plaques observed were characteristic of each plaque variant of WEE virus.

V. HOST CELLS OTHER THAN CHICK EMBRYO.

Characterization of SP-6 and LP-7 viruses was made only by plaques produced on monolayer cell cultures of primary chick embryo. As additional evidence to ascertain whether SP-6 and LP-7 plaques were genetically associated with the viruses, plaque formation with particular reference to the morphological differences was tried using other cultured cells

infected with SP-6 and LP-7 viruses. However, virus synthesized from chick embryo cells failed to develop plaques on monolayer cell cultures of HeLa, L, FL, mouse embryo, hamster kidney, and guinea pig kidney. Consequently, susceptibility studies were carried out with the various cultured cells infected and maintained in culture tubes. Also attempts were made to select SP-6 and LP-7 viruses capable of infecting these cells.

A. Susceptibility of Other Cell Lines.

The cells described above were grown in screw-cap culture tubes and "infected" with each mutant of WEE virus at a multiplicity of 5. After an hour of incubation at 25 C, the monolayer cell cultures were thoroughly washed with small volumes of growth medium. When the culture tubes were incubated at 37 C for 48 hours in growth medium, the cells were observed microscopically for indications of cellular degeneration. Aliquot samples of the expended growth fluid were titrated by the plaque assay technique on monolayer cultures of primary chick embryo cells. Three successive passages of the fluid were made in the respective cells. The results of the experiments from the first and third passages are shown in Table 8.

The monolayer cells of HeLa, L, and guinea pig kidney infected with each mutant virus demonstrated a slight degree of cytopathogenicity as compared to normal control cells.

TABLE 8

Susceptibility of various cell culture lines to infection by SP-6 and LP-7 mutants of WEE virus.

	Passage No. 1		Passage No. 3	
Cell Cultures	Cell Degeneration	PFU/cell	Cell Degeneration	PFU/cell
HeLa	±[a]	1[c]	-	0
FL	-[b]	0	-	0
L	±	1[c]	-	0
Mouse Embryo	-	0	-	0
Hamster Kidney	-	0	-	0
Guinea Pig Guinea	±	2.6 (LP-7) 2.0 (SP-6)	+ +	20 (LP-7) 20 (SP-6)

[a] Degree of cell degeneration

[b] Cells normal

[c] Approximately 1 PFU/5-10 cells

In the first passage less than 1 infective virus particle were released per HeLa and L cells and approximately 2 by guinea pig kidney cells after 48 hours of incubation. The monolayer cells of FL, mouse embryo, or hamster kidney were morphologically undistinguishable from normal control cells. Furthermore, infective virus particles in the fluid were not detectable by the standard plaque assay technique using monolayer cell cultures of chick embryo.

The HeLa and L cells of the third passage were morphologically normal as were FL, mouse embryo, and hamster kidney cells. Similarly, infective virus particles were not detectable in growth fluids from each of the culture tubes. On the other hand, guinea pig kidney cells of the third passage were completely degenerated after 48 hours of incubation. It was also found that approximately 20 PFU were released per cell infected with either SP-6 or LP-7 virus.

B. Plaque Development on Chick Embryo Cell Cultures.

The SP-6 and LP-7 mutant viruses from the different infected cell lines previously described were titrated by the plaque assay technique on monolayer cultures of primary chick embryo cells. The plaques developed were measured after 54 hours of incubation. As shown in Table 9 the plaque dimensions were characteristic of each mutant.

TABLE 9

Plaque development on monolayer of primary chick embryo infected with SP-6 and LP-7 viruses synthesized from other cell lines.

Virus Source	Plaque Diameter in mm at 56 hrs	
	SP-6	LP-7
HeLa (passage 1)	2	8
L (passage 1)	2	8
Guinea pig kidney (passages 1, 2, 3)	2	8

C. Plaque Formation by Other Cell Lines.

Recently, Fuerst (1961) reported that infected monolayer cultures overlayed with 2.5 ml of agar overlay medium followed by a second layer of liquid growth medium formed plaques with distinct outlines. This technique enabled the liquid layer to be changed after a certain length of time, thus enabling the monolayer cells to be maintained in a suitable cultural environment throughout the period of incubation.

Liebhaber and Takemoto (1961b) found that the addition of DEAE dextran in the overlay medium enabled certain strains of coxsackie virus to produce plaques on monolayer cultures of monkey kidney cells. These coxsackie viruses were normally unable to develop plaques under the standard procedures. Therefore, these methods were incorporated in the

experiments and the results obtained are recorded in Table 10.

Plaques were not observed in monolayer cultures of HeLa, L, and guinea pig cells infected with SP-6 and LP-7 viruses. However, third passage viruses by infected guinea pig cells were able to produce plaques on monolayer cultures of guinea pig kidney cells and only when the agar overlay medium contained DEAE-Sephadex. The SP-6 plaques measured 1 mm in diameter and LP-7 plaques 3 to 4 mm.

VI. ADSORPTION.

If SP-6 virus adsorbed to chick embryo cells less efficiently or at a rate slower than LP-7 virus, the diameter of the SP-6 plaques could be smaller. Therefore, the adsorption or attachment rates of SP-6 and LP-7 viruses were determined.

Confluent cell monolayers of primary chick embryo were washed three times with 2 ml volumes of 25 C PD solution before adding 0.2 ml of virus suspensions containing 90 PFU. The infected monolayers were immediately placed in the 25 C incubator. At various time intervals, the monolayer cultures were washed twice with 2 ml volumes of PD before the addition of agar overlay media.

The results of the adsorption experiment, as shown in Fig. 6, indicated that there were no differences in the rate of adsorption between the two mutants on monolayer cultures of primary chick embryo cells. Approximately 25% of the virus

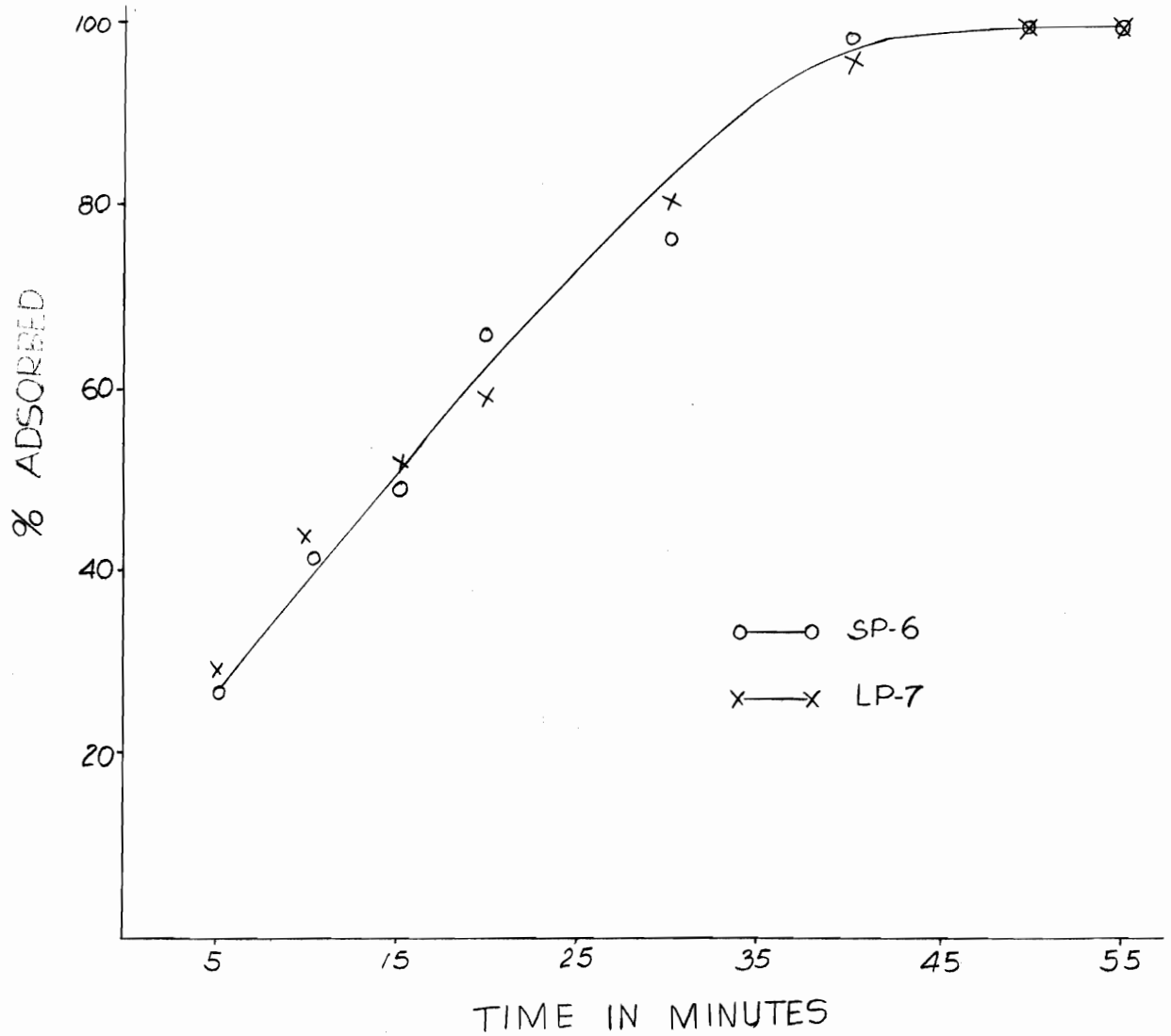

FIG. 6. Adsorption of SP-6 and LP-7 mutants of WEE virus on monolayer cell cultures of primary chick embryo.

had adsorbed to the monolayer cells within 5 minutes and 85% within 35 minutes. Adsorption was virtually complete for both mutant viruses after 50 minutes of incubation. Attempts to determine attachment rate in a cell suspension system were not successful.

VII. STABILITY.

In the case of SP-6 and LP-7 viruses, stability could be a factor affecting plaque development. Infection of surrounding cells would be difficult to achieve if the virus released from an infected cell is readily inactivated. Therefore, the stability between the two mutant viruses was determined.

Virus suspensions were diluted in 37 C PBS (pH 7.2) and growth medium. At various time intervals aliquot samples were removed, diluted with equal volume of 4 C growth medium, and titrated by the plaque assay technique.

The stability or rate of inactivation between SP-6 and LP-7 viruses at 37 C in the suspending media were found to be similar. The result of the stability experiments in PBS is illustrated in Fig. 7, which shows that the half-life of each mutant virus was approximately 20 minutes. The pH of the buffer was found to be critical and influenced the stability of the mutants. At pH 6.8 SP-6 and LP-7 viruses were completely inactivated within 5 minutes.

Both mutants demonstrated increased resistance to inactivation by 37 C temperature in the presence of protein. For

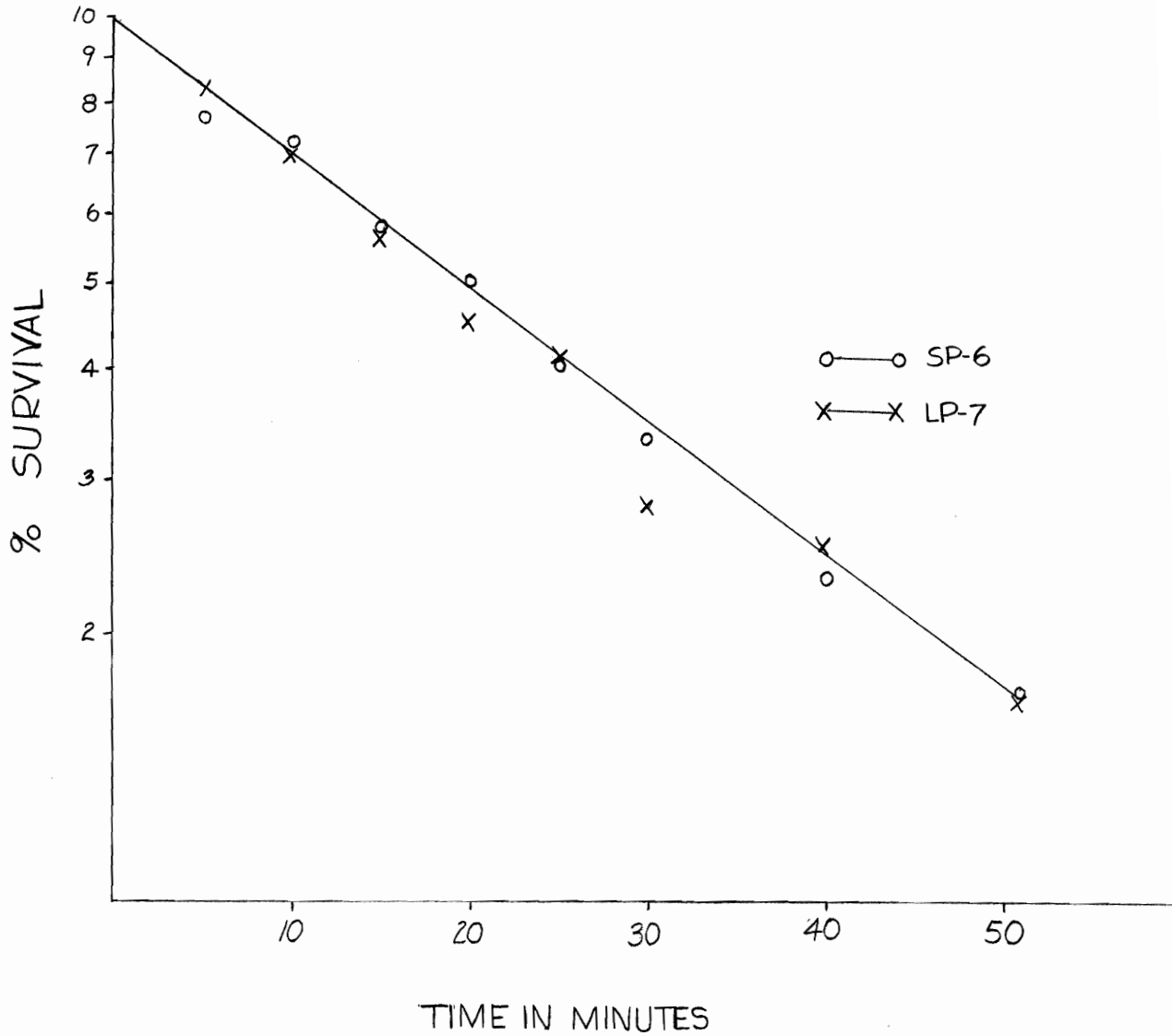

FIG. 7. Stability at 37 C of SP-6 and LP-7 mutants of WEE virus suspended in PBS (pH 7.2).

example, in growth medium containing 3% calf serum and 0.4% lactalbumin hydrolysate the half-life or 50% inactivation was 4 hours (Fig. 8).

VIII. GROWTH CURVE STUDIES.

Other factors responsible for the differences in plaque diameters formed by SP-6 and LP-7 viruses could have been due to lag period or total virus released per infected cell in a monolayer cell culture. Therefore, growth curve experiments were made with infected cells maintained under various conditions.

It was earlier mentioned that neutral red was added 36 hours later to the monolayer cell cultures infected with SP-6 virus for the quantitative plaque assay determination. An important factor to be considered is that in preliminary experiments for quantitative assay purposes, no differences in plaque counts were noted between 42 and 54 hour SP-6 plaques. In addition, the plating efficiency of each mutant virus was found to be similar. Therefore, any error in plaque counts involved in the data should be distributed equally.

A. Cell Monolayer System with Growth Medium.

Monolayers of cell cultures grown in 16 oz prescription bottles were thoroughly washed with PD solution. Based on an average cell population from 3 different monolayer cultures, cells were infected with a multiplicity of 10. After 1 hour of incubation at 25 C, the infected monolayers were

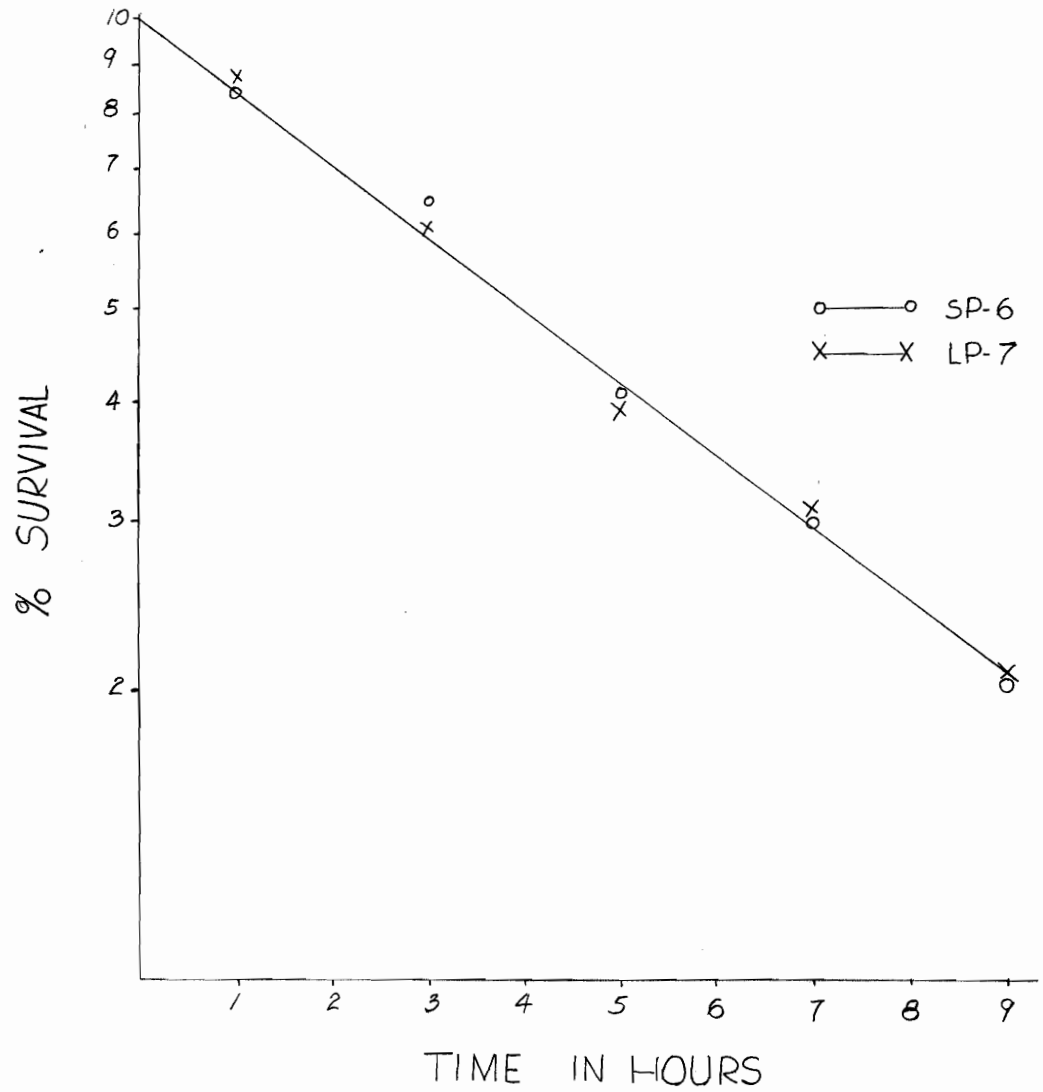

FIG. 8. Stability at 37 C of SP-6 and LP-7 mutants of WEE virus suspended in growth medium.

washed 3 times with 5 ml volumes of growth medium. At time zero 50 ml of 37 C growth medium were added to each bottle. At designated time intervals, 0.1 ml samples were withdrawn, added to separate tubes containing 0.9 ml of 4 C growth medium, and placed in the freezer. The lost volume was immediately replaced with 37 C growth medium and the infected cultures incubated at 37 C. Virus titer was titrated by the plaque assay technique.

The results of the experiment, depicted as a graph in Fig. 9, indicated that release of newly synthesized virus by LP-7 mutant infected cells was detectable as early as 1½ hours after incubation. In contrast, a lag of approximately 3 hours was evident in the release of infective particles by cells infected with SP-6 virus.

Accelerated release of infective virus particles was evident by cells infected with either mutant virus to 8 hours, after which further increase of virus titer in the suspension medium was not detectable.

Significant differences, however, were noted in relation to accumulated virus particles released per cell at the termination of the incubation period by cells infected with each mutant virus. Calculating the ratio of infective virus at 12 hours to average cell population per bottle showed that each SP-6 virus infected cell released 100 PFU in contrast to 2000 PFU by LP-7 virus infected cells (Table 10).

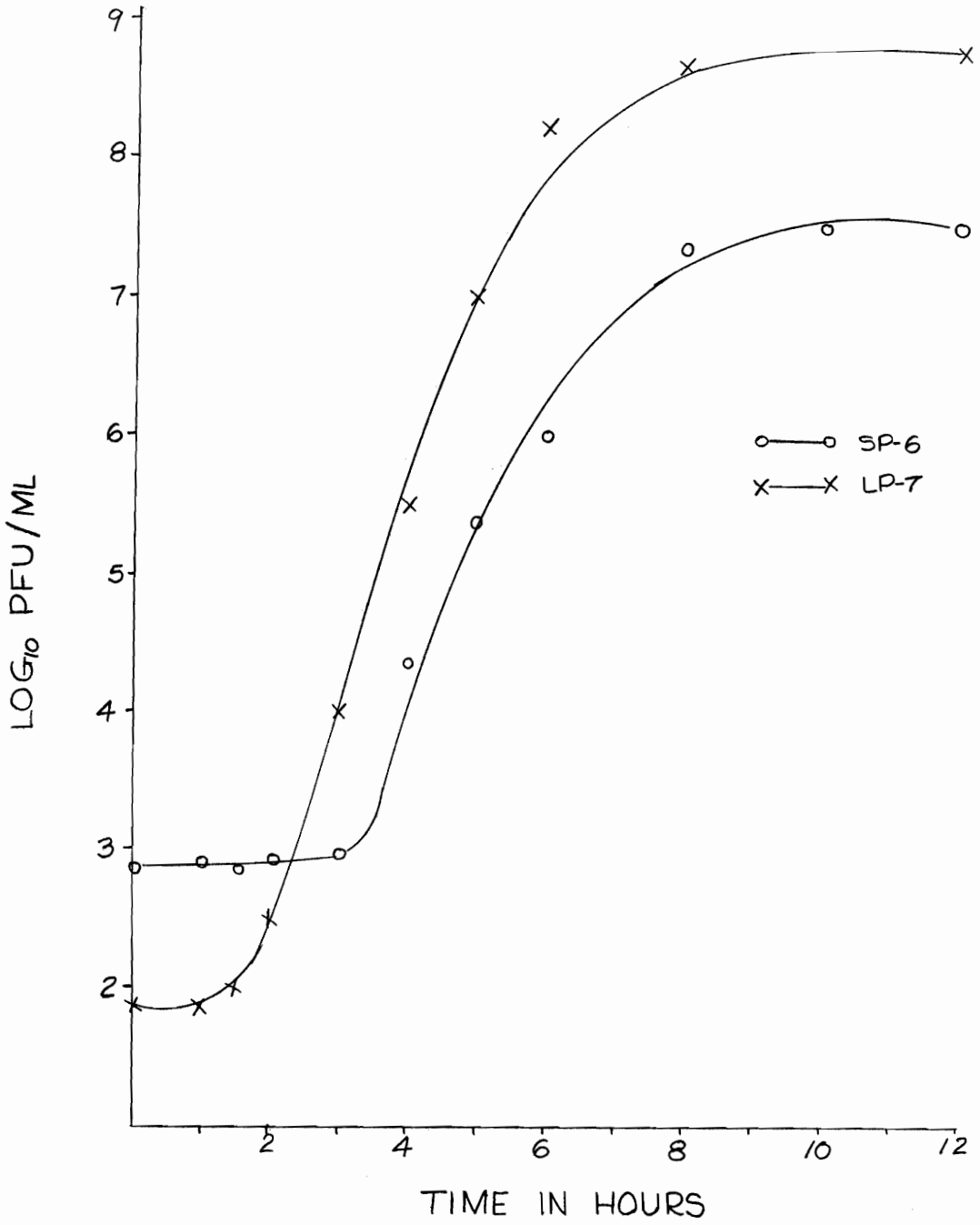

FIG. 9. Growth curve studies of SP-6 and LP-7 mutants of WEE virus infected to monolayer cell cultures of primary chick embryo. Infected monolayers incubated with growth medium at 37 C.

TABLE 10

Total number of PFU released per infected cell in growth curve experiments at 12 hours.

Experimental System	Virus Infected Cells	Number Cells/ml	Virus Titer PFU/ml	PFU/cell
Monolayer (growth medium)	SP-6	3×10^5	3×10^7	100
	LP-7	3×10^5	6.2×10^8	2000
Monolayer (Agar overlay extract medium)	SP-6	3.8×10^5	6×10^6	16
	LP-7	3.8×10^5	5.9×10^8	1500
Cell suspension (growth medium)	SP-6	6.9×10^2	1×10^5	140
	LP-7	2.7×10^2	3×10^5	1100

B. Cell Monolayer System with Agar Overlay Medium Extract.

To ascertain whether the agar inhibitor affected virus synthesis, agar overlay medium was frozen overnight at -20 C as described by Takemori and Nomura (1961). When thawed the extract was separated by centrifugation, equilibrated in 37 C water bath, and added to monolayer cultures of chick embryo cells previously infected with each mutant at a multiplicity of 10. The initial infection of the cells was made with the virus suspended in normal growth medium. The remaining portions of the experiment were conducted as described in the previous section.

As shown in Fig. 10, similar results were obtained in relation to temporal differences and accelerated virus release. A significant difference was observed, however, on total virus released per cell at the termination of the incubation period (Table 10). Approximately 1500 PFU were released per cell infected with the LP-7 mutant, but only 16 PFU were released by each SP-6 virus infected cell.

C. Cell Suspension System with Growth Medium.

The monolayer cell cultures were washed and infected with each mutant virus as described. However, after washing the infected monolayer cultures with PD, 1 ml 0.05% trypsin was added to each monolayer culture to obtain a free cell suspension. Approximately 2 X 10^5 cells were added to a "Bellco" spinner flask containing 195 ml of 37 C growth medium

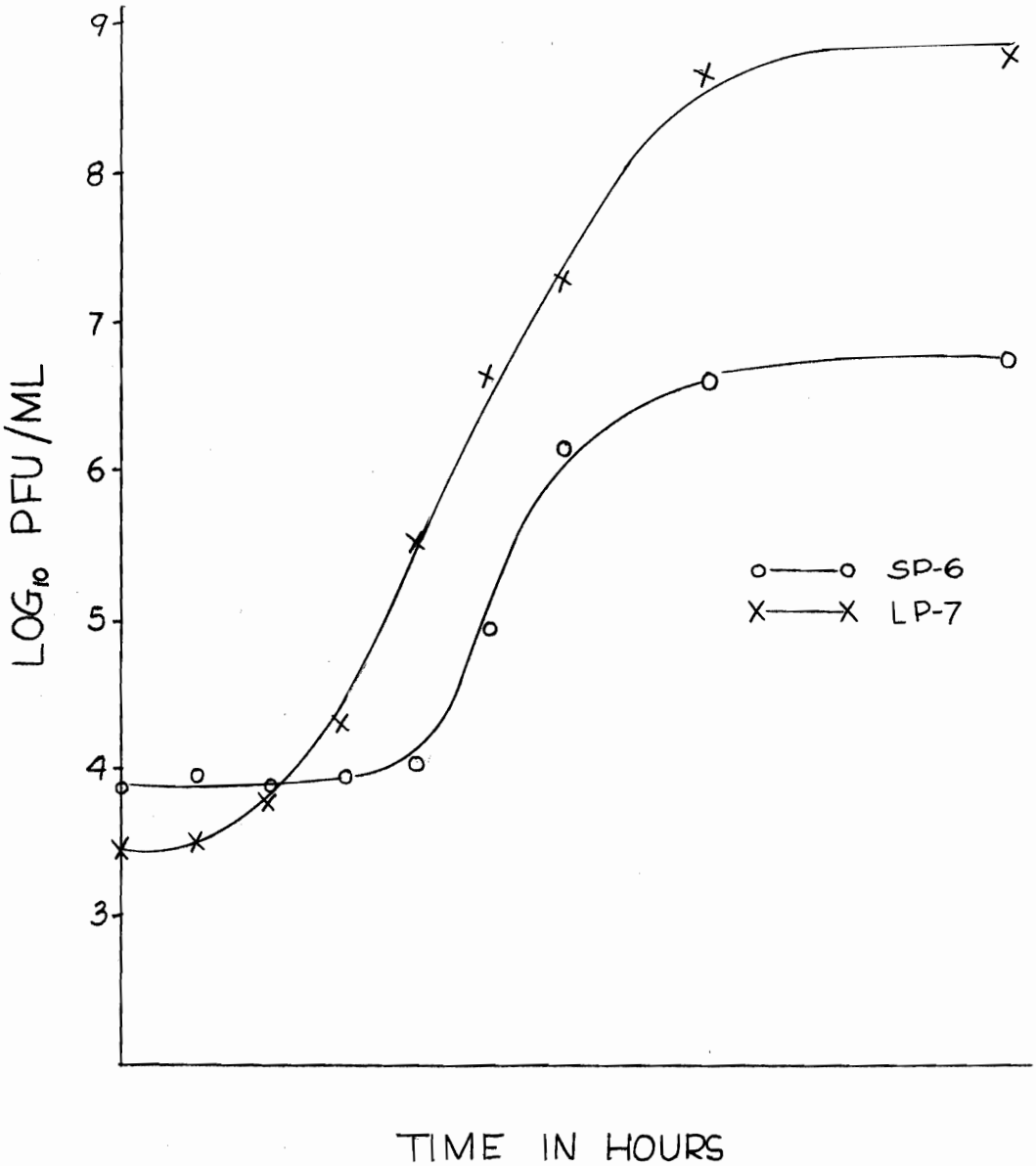

FIG. 10. Growth curve studies of SP-6 and LP-7 mutants of WEE virus infected to monolayer cell cultures of primary chick embryo. Infected monolayers incubated with agar overlay medium extract at 37 C.

and the volume brought up to 200 ml. At various time intervals, 5 ml samples were removed, centrifuged, and the supernatant frozen. The spinner flasks were maintained at 37 C throughout the experiment in a constant temperature water bath.

As an efficient means of calculating viable and infected cells per ml, an infective center assay technique was utilized.

As soon as infected cell suspensions were added to and dispersed in the 200 ml volume of growth medium, an aliquot sample from each spinner flask was added to monolayer cultures of primary chick embryo cells. One hour was allowed for cells and unadsorbed virus to attach to the monolayer cells at 37 C. One ml of overlay medium was carefully added to each monolayer cell culture followed by 3 more mls of overlay medium after the initial overlay had solidified. The supernatant from a centrifuged sample removed from the spinner flask was similarly titrated. The difference in the number of plaques between the two samples indicated the concentration of infected cells in a given volume.

The data obtained from this experiment and plotted in Fig. 11 again showed a difference in lag period of virus release by cells infected with each mutant virus. Calculation of PFU/cell at 12 hours gave results which were nearly identical with the experiment where infected monolayer cells were incubated with growth medium. The cells infected with LP-7

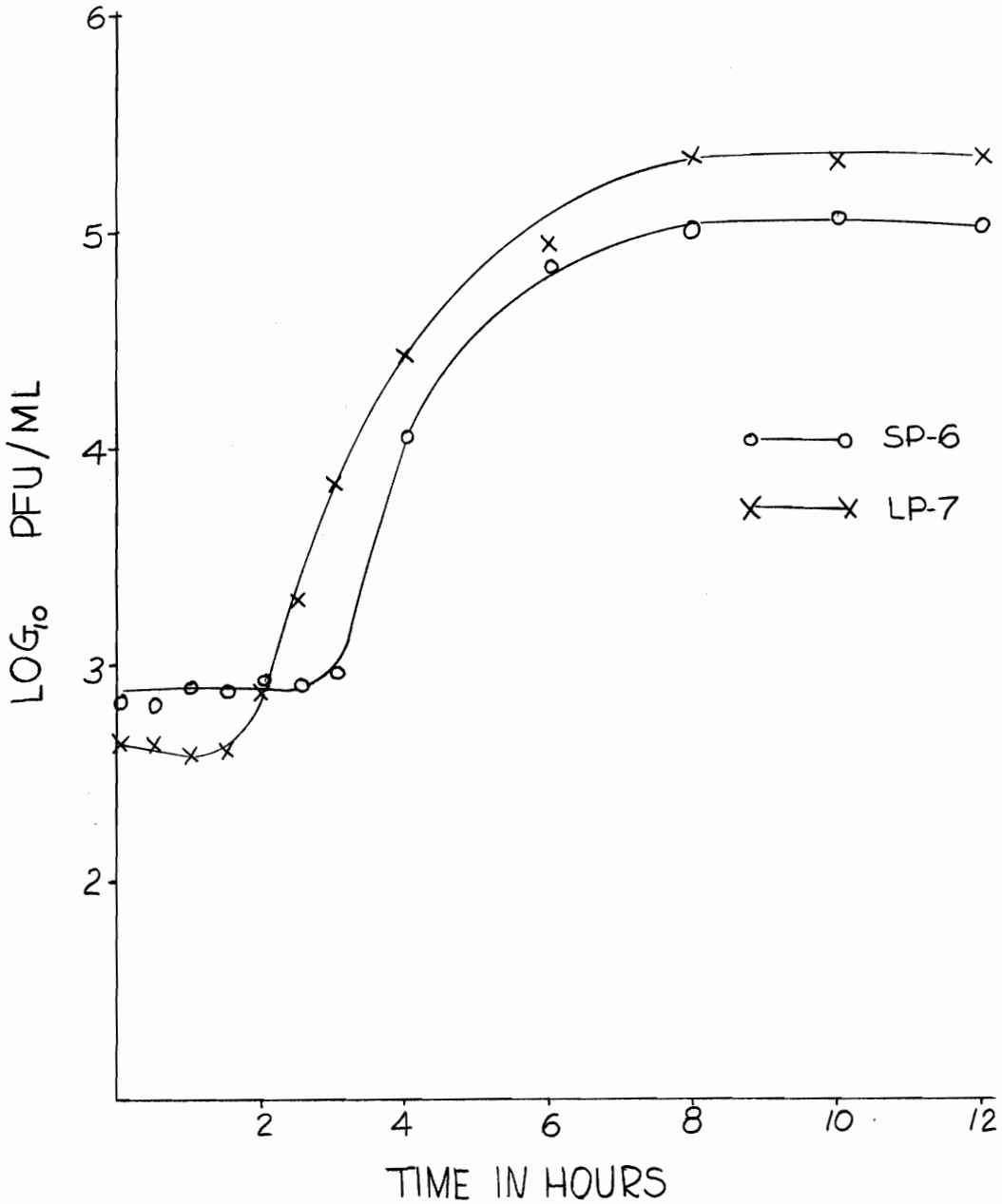

FIG. 11. Growth curve studies of SP-6 and LP-7 mutants of WEE virus infected to monolayer cell cultures of primary chick embryo and infected cells incubated as suspension with growth medium at 37 C.

virus, however, released somewhat fewer virus (Table 10).

IX. SUSCEPTIBILITY OF SUCKLING MICE AND "WET" CHICKS.

Cockeral chicks hatched within 12 hours or commonly referred to as "wet" chicks were separated into lots of 5, injected subcutaneously in the dorsal region between the wings with 0.1 ml of growth medium containing a known PFU titer of each mutant virus. Chick mash feed and water were given to the chicks two days later. Sexing of the chicks were based on the judgement of an experienced "chick sexer."

Suckling mice between 14 to 16 days in age were each injected intracerebrally with 0.025 ml of the inoculum and allowed to remain with a lactating mouse. All chicks and mice surviving the experiments were killed, autoclaved, and discarded.

The results of the experiments shown in Tables 11 and 12 indicate that both SP-6 and LP-7 viruses were highly lethal for "wet" chicks and suckling mice injected through the routes described. At least 2 PFU of each mutant virus were sufficient to cause death in the two different animals.

Death, however, generally appeared to occur sooner in animals experimentally infected with the LP-7 virus as compared to those infected with the SP-6 virus.

TABLE 11

Susceptibility of "wet" chicks challenged with SP-6 and LP-7 viruses. Titers of each mutant virus in the inocula are based on PFU.

	SP-6 VIRUS			
PFU	2*	3	4	5
480	12/20	17/20	20/20	
48	6/20	12/20	20/20	
17.6	11/20	15/20	20/20	
8.8	6/20	14/20	20/20	
4.4	8/20	16/20	20/20	
2.2	8/20	16/20	19/20	20/20
1.1	3/20	13/20	13/20	13/20

	LP-7 VIRUS			
PFU	2	3	4	5
600	20/20			
60	19/20	20/20		
13	18/20	18/20	20/20	
6.5	20/20			
3.3	20/20			
1.6	20/20			
0.8	10/20	11/20	11/20	12/20

* days after challenge.

TABLE 12

Susceptibility of 14 day old mice challenged with SP-6 and LP-7 virus. Titers of each mutant virus in inocula based on PFU.

		SP-6 VIRUS		
PFU	2*	3	4	5
7500	1/8	8/8		
750	0/8	5/8	8/8	
75	0/8	5/8	7/8	8/8
7.5	0/8	0/8	4/8	7/8
2	0/8	0/8	3/8	8/8

		LP-7 VIRUS		
PFU	2	3	4	5
7000	7/8	8/8		
700	2/8	8/8		
70	1/8	6/8	8/8	
7	6/8	8/8		
2	3/8	8/8		

* days after challenge

DISCUSSION

The progeny viruses from the isolates, SP-6 and LP-7, consistantly produced plaques with diameters of 2 mm and 8 mm respectively on monolayer cell cultures of primary chick embryo. Both of these viruses were considered to be mutants since the plaques formed by the parent WEE virus measured 5 mm in diameter.

Reeves (1961) found that all field isolates of WEE virus from mosquitoes and birds produced characteristically large plaques on primary isolation and included a very small number of viruses which produced smaller plaques. The strains of WEE virus obtained from Rocky Mountain Laboratory (RML) were isolated from mosquitoes and had been passed through mice. However, when these strains were titrated by the plaque assay technique, strain 85 produced plaques which were morphologically similar to those of SP-6 virus. Although a few smaller plaques were formed on all monolayer cell cultures infected with the other RML strains of WEE virus, the diameter of the larger plaques measured only 5 mm in diameter. Therefore, it seems that mutation toward small plaque formation by WEE virus can occur in cell cultures as well as in nature. Furthermore, the plaque diameter of 5 mm perhaps may be characteristic of the wild-type WEE virus in nature. Whether mutation toward large plaque formation, as demonstrated by the LP-7 plaques, does occur in nature can only be decided by titrating a number of

WEE viruses isolated directly from nature by the plaque assay technique.

Experimental results from the neutralization tests indicated that SP-6 and LP-7 viruses were serologically related since both viruses were readily neutralized by standard WEE virus antiserum and by antisera prepared against each virus in chicks. The results indicating the higher neutralizing titer of LP-7 virus antiserum may be questioned, since preparation of antiserum from another lot of chicks for confirmatory purposes was not done. However, Reeves et al. (1958) found only 1 of 11 virus-positive pool suspensions, prepared from mosquitoes isolated during January to March, produced a significant antibody response in inoculated chickens. Positive pools prepared from summer isolates induced high antibody response almost consistently. Possible reasons for the higher antibody response may be that during the summer months infected mosquitoes may possess a higher virus titer, or virus synthesized by the infected mosquito tissues may be more antigenic. These reasons may also be the case with SP-6 and LP-7 viruses. The results from the growth curve experiments have shown that cells infected by the LP-7 virus released considerably more virus than those infected by the SP-6 virus. Therefore, chicks infected by the LP-7 virus may have demonstrated a higher level of viremia, which in turn may have elicited the higher antibody response.

Three intracerebral passages of SP-6 and LP-7 viruses in mice failed to alter the phenotypic properties responsible for the development of different sized plaques on monolayer cell cultures of chick embryo. The results obtained were similar with viruses from infected HeLa, L, and guinea pig kidney cell cultures. Finally, plaques developed on monolayer cell cultures of guinea pig kidney demonstrated differences in diameters although the plaques produced by each mutant virus were much smaller as compared to those formed on chick cells. The overlay medium prepared from purified and unpurified agar was found to have no affect on the development of plaque size produced by each mutant virus.

Takemori et al. (1957) isolated small plaque mutants of type 1 poliovirus which were insensitive to an inhibitor found in certain normal bovine serum prepared at specified concentrations in the overlay medium. Dulbecco and Vogt (1955) also obtained a small plaque mutant of poliovirus from an infected monolayer culture of monkey kidney cell overlayed with a medium containing chick embryo extract, but the mutant plaque was undistinguishable from the wild parent virus when the optimal nutrient, monkey serum, was added to the overlay medium. Calf serum in concentrations of 1.5, 3, 5, and 10% in the overlay medium failed to alter the plaque size characteristic of each WEE virus mutant. Horse serum at similar concentrations, however, suppressed the plaque dimension of each

mutant virus as much as 50%, and previous examination of the serum indicated the absence of any neutralizing activity. At the moment nutritional incompatability by chick cells to calf serum cannot be ascertained as a factor responsible for the differences in plaque size until the affect of other nutritional sources as chicken serum or chick embryo extract have been determined. However, it appears that horse serum was suboptimal for plaque formation by both mutant viruses as compared to calf serum.

Recently, Liebhaber and Takemoto (1961b) reported that an inhibitor naturally occurring in agar was responsible for the minute plaque formation by a mutant of murine encephalomyocarditis (EMC) virus. These investigators found that the addition of DEAE dextran in the agar overlay medium enabled the mutant virus to develop plaques undistinguishable in dimension from the wild-type EMC virus. By adding DEAE-Sephadex A-50 to the agar overlay, since the dextran was not commercially available, it was found that, although the LP-7 plaques were not affected, a substance was at least partially responsible for the production of the smaller plaques produced by the SP-6 virus. Apparently, the substance is the inhibitor described by Liebhaber and Takemoto (1961b).

DEAE-Sephadex sterilized separately from agar was not capable of neutralizing the inhibitor, whereas autoclaving the anion exchanger with the agar solution proved to be most

efficient. A final concentration of 50 ug/ml of DEAE-Sephadex in the agar overlay medium was found to be adequate in suppressing the inhibitory activity. The agar lost the solidifying properties with higher concentrations of the anion exchanger.

Since agar was found to contain an inhibitor partially responsible for suppressing SP-6 virus plaques, methylcellulose of two different grades, based on viscosity, was substituted. Plaques produced by each mutant virus under overlay media containing either sterilized Methocel 100 cps or 4000 cps were similar in diameters to those observed where the agar overlay medium contained horse serum. Unautoclaved methylcellulose suppressed the plaques of both mutant viruses where the SP-6 plaques were barely visible. These results are unexplicable at the moment. However, the experimental results suggest that viscosity was not a factor in the differences of plaque diameter, since methylcellulose overlay media did not demonstrate the solidifying properties exhibited by agar overlay media, and neutral red solution was completely diffused throughout the methyl cellulose overlay medium within a few minutes after addition.

The development of SP-6 and LP-7 plaques on monolayer cell cultures of guinea pig kidney was only possible when the agar overlay medium contained DEAE-Sephadex. Liebhaber and Takemoto (1961) similarly were able to obtain plaques from

ECHO viruses (types 4, 5, and 6) in monkey kidney cultures by the addition of DEAE dextran to agar overlay medium, whereas control plates without the dextran failed to demonstrate plaques. The plaques produced on cultures of guinea pig kidney cells were considerably smaller but the differences in diameters between plaques produced by each mutant virus remained proportionately unchanged. Nevertheless, the inhibitor in agar must have been involved since infected monolayer cultures of guinea pig kidney overlayed without added DEAE-Sephadex in the agar overlay medium failed to develop plaques.

The distinct plaques formed on infected guinea pig kidney cells initially covered with small volumes of agar overlay medium followed by 3 ml of liquid growth medium can be explained. After 48 hours of incubation at 37 C, the overlay media in all plates were relatively acidic. By changing the growth medium of these plates, the viable or uninfected cells were able to metabolize actively, thus enabling the cells to be readily discernible by the vital stain.

When monolayer cultures of guinea pig kidney, mouse embryo, hamster kidney, HeLa, FL and L cells were infected with each mutant virus and overlayed with or without added DEAE-Sephadex, plaques failed to develop. Therefore, these cells were grown in tubes, infected with each mutant virus, and the expended fluid tested for the presence of synthesized virus. The cells were also observed for degeneration micro-

scopically. Slight cellular degenerations were observed in the infected HeLa, L and guinea pig cells and low concentrations of each mutant virus were detected in the fluid. However, only SP-6 and LP-7 viruses from infected guinea pig cells could be passaged further. Upon additional passages of each mutant virus in normal guinea pig cells, the viruses demonstrated higher capabilities of infecting the guinea pig kidney cells. Without doubt only mutant SP-6 and LP-7 viruses capable of infecting guinea pig kidney cells were able to be selected.

Lockart and Groman (1958) reported that plaques measuring 1 mm in diameter were formed on monolayer cultures of chick embryo fibroblasts infected with an inoculum containing an overwhelming majority of 37 C inactivated WEE virus but normal plaque size of 5 mm was attained with progressively larger dilutions. Similar results were obtained when monolayer cultures of chick embryo cells were incubated overnight with inactivated SP-6 virus and later infected with either SP-6 or LP-7 viruses. Plaques observed were considerably smaller in diameter as compared to the controls not exposed to the inactivated virus.

Suspensions of SP-6 virus progressively diluted as described by Lockart and Groman (1958) and titrated on chick embryo cells failed to develop plaques. When monolayer cultures were incubated overnight with the diluted suspension

prior to infection with SP-6 and LP-7 virus, the usual plaque sizes characteristic of each mutant virus were seen. Finally, normal plaques were also produced from suspensions containing SP-6 or LP-7 viruses synthesized within an hour by infected chick cells. These results indicated that homologous interference was not involved in the differences of plaque diameters produced by SP-6 and LP-7 viruses on monolayer cell cultures of chick embryo.

Both mutant viruses were found to adsorb at the same rate on monolayer cultures of chick embryo cells. The results from the stability experiments also indicated that the inactivation rates at 37 C of the two mutants were identical with a half-life of 20 minutes in PBS and 4 hours in growth medium. Therefore, stability and rate of adsorption were factors not responsible for the production of SP-6 and LP-7 plaques.

In each of the growth curve experiments similar results were observed in the differences of lag time of virus release and total virus released per cell. When LP-7 virus infected cells were maintained in suspension or as monolayers with growth medium or agar overlay medium extract, a lag period of 1½ hours was evident before newly synthesized virus was detectable. Cells maintained under identical conditions but infected with SP-6 virus required approximately 3 hours to release virus. Maximum virus release was attained within 8

to 10 hours by all infected cells, but total virus yielded per cell was different in each case.

The experimental results obtained from the study of infected cells maintained as monolayer cultures showed that each cell infected with SP-6 virus released 100 PFU when incubated with growth medium in contrast to 16 PFU with agar overlay medium extract. On the other hand 2000 PFU and 1500 PFU/cell were released by LP-7 virus infected cells incubated with the respective media.

In view on the experimental results presented, possible reasons for the differences in plaque diameters formed by SP-6 and LP-7 viruses on monolayer cultures of primary chick embryo cells will be speculated.

The LP-7 infected cell was able to infect the adjoining cell 2 hours earlier than the SP-6 virus infected cell. In the presence of the inhibitor naturally occurring in agar, SP-6 virus infected cells released considerably fewer particles. Consequently, infection may have been initiated later in normal cells if the virus particles were released from the infected cell at the region of initial infection rather than the opposite side of the cell or the area adjoining normal cells. Furthermore, relative to the size of the cell, virus released from the region of initial infection had to migrate a considerable distance to infect adjoining normal cells. These reasons may account for the "irregular" edges demonstrated by the SP-6 plaques.

When the inhibitor was neutralized by DEAE-Sephadex, SP-6 virus infected cells were able to release more virus particles which increased the chances of infecting adjoining cells. The LP-7 virus infected cells, however, still released approximately 10 to 20 times more virus per cell. Therefore, coupled with the lag period of virus release, the SP-6 plaques were still unable to attain the diameter produced by the LP-7 plaques.

Other factors are undoubtedly contributing to the formation of the different plaques sizes produced by SP-6 and LP-7 viruses. Certain of these can only be determined after the development of better techniques as well as after obtaining further knowledge of the host cell-virus interaction.

In addition to the experiments discussed, unrelated studies on susceptibility showed that both SP-6 and LP-7 viruses were highly lethal for suckling mice and "wet" chicks through intracerebral and subcutaneous injections respectively. Reeves et al. (1958) found that many WEE virus isolated directly from C. tarsalis in nature during the winter months demonstrated low susceptibility for adult mice. These viruses from the mosquitoes were able to kill adult mice by intracerebral injection only after the virus had been through 3 to 6 successive passages in embryonated eggs, and a few of the virus isolates were unable to kill embryonated eggs until further passages.

It is tempting to speculate that less virulent strains of WEE virus do exist in nature. For example, Hammon and Reeves (1947) and LaVeck et al. (1955) have shown that in an endemic area where WEE virus can be isolated readily from mosquitoes, a number of persons possessed circulating neutralizing antibodies against WEE virus, although none of these individuals have had any history of encephalitis. On the other hand, outbreaks of WEE virus infection in Saskatchewan resulted in a number of human cases with clinical symptoms (Gareau and Fulton, 1941). Mutation may account for the differences in virulence demonstrated by WEE virus in an endemic area.

SUMMARY

Two mutant viruses were isolated from a culture of Western equine encephalitis (WEE) virus strain W-4. Plaques developed by mutant virus SP-6 measured 2 mm in diameter on monolayer cell cultures of primary chick embryo after 54 hours of incubation compared to 8 mm by the mutant virus LP-7. Studies were made to determine the physical factors responsible for the differences in plaque diameters produced by the mutant viruses. Other properties of these two mutant viruses were also investigated. The experimental results are summarized as follows:

1. Standard WEE virus antiserum readily neutralized SP-6 and LP-7 viruses. Cross neutralization was also observed with antisera prepared against each mutant virus.

2. Viruses of both mutants from infected HeLa, L, or guinea pig kidney culture cells and from infected mice brain retained their phenotypic characteristics on monolayer culture of chick embryo cells.

3. Varying the concentration of calf serum from 1.5 to 10% in the agar overlay medium resulted in the production of plaques with diameters characteristic of each mutant virus. Horse serum in similar concentrations caused the plaque diameters produced by each mutant to be suppressed by 50%.

4. No differences in plaque diameters were observed with agar overlay medium prepared from either purified or unpurified

agar.

5. Neutralization of an inhibitor in agar with DEAE-Sephadex resulted in the formation of SP-6 plaques measuring 5 mm in diameter but LP-7 plaques measured the usual 8 mm.

6. Methylcellulose was substituted for agar in the overlay medium. The diameters of SP-6 and LP-7 plaques were proportionately smaller.

7. Monolayer cultures of guinea pig kidney cells infected with selected SP-6 and LP-7 viruses produced plaques with proportionately smaller diameters. Plaques were demonstrable only when the agar overlay medium contained DEAE-Sephadex.

8. Homologous interference was not responsible for the suppressed diameter of SP-6 plaques.

9. Rate of adsorption on monolayer cultures of chick embryo cells was similar for both mutants.

10. Both mutant viruses were suspended in phosphate-buffered saline (PBS) and in growth medium. The inactivation rates were similar at 37 C for both mutants. The half-life activities were found to be 20 minutes in PBS and 4 hours in growth medium.

11. Three separate growth curve experiments were performed with chick cells infected with each mutant virus. Infected cells incubated a monolayers with growth medium or agar overlay medium extract or in suspension demonstrated similar results in relation to the lag period of virus releases. LP-7 infected cells released newly synthesized virus within 1½ hours

whereas, a lag of 3½ hours was evident for SP-6 infected cells. At 12 hours significant differences were found in total virus released per cell infected with each strain and incubated under each of the described conditions.

Based on these experimental results possible factors responsible for the differences in plaque diameters produced by SP-6 and LP-7 viruses were discussed.

Additional experiments made concerning the two mutant viruses are summarized:

1. Attempts to select mutants from SP-6 and LP-7 viruses which were able to readily infect HeLa, L, FL, mouse embryo, and hamster kidney were not successful.

2. Each mutant virus was highly lethal for "wet" chicks and suckling mice injected by subcutaneous and intracerebral routes respectively. Less than 2 plaque-forming-units (PFU) was found sufficient to kill the experimental animals within 5 days.

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PLAQUE MUTANTS OF WESTERN EQUINE ENCEPHALITIS VIRUS

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Two mutant viruses differing in the production of plaque morphology were isolated from a culture of Western equine encephalitis (WEE) virus strain W-4, which was propagated from an isolated plaque originally measuring 5 mm in diameter on monolayer cell cultures of primary chick embryo after 54 hours of incubation. Plaques developed by the mutant virus SP-6 were 2 mm in diameter compared to 8 mm by the mutant virus LP-7. Studies were made to determine what physical factors were responsible for the differences in plaque diameters produced by the mutant WEE viruses. Other properties of these two mutants were also investigated.

Both of the mutant viruses were readily neutralized by standard WEE virus antiserum obtained from the Communicable Disease Center, Atlanta, Georgia. Cross neutralization was also observed with antisera prepared against each mutant virus.

The SP-6 and LP-7 viruses propagated by infecting HeLa, L, or guinea pig culture cells and from infected mice brains retained their phenotypic characteristics on monolayer of chick embryo cells.

Varying the concentration of calf serum from 1.5 to 10% in the agar overlay medium resulted in the production of plaques with diameters characteristic of each mutant virus. Horse serum in similar concentrations caused the plaques diameters produced by each mutant to be suppressed by 50%.

Differences in plaque size were not observed with agar

overlay media prepared from either purified or unpurified agar. However, neutralization of an inhibitor in agar with DEAE-Sephadex resulted in the formation of SP-6 plaques measuring 5 mm in diameter. The LP-7 plaques produced were 8mm. When methylcellulose was substituted for agar in the overlay medium. the SP-6 and LP-7 plaques that developed were considerably smaller in diameter proportionately.

Monolayer cultures of guinea pig kidney cells infected with selected SP-6 and LP-7 viruses produced plaques with proportionately smaller diameters, but plaques by these cells were only demonstrable when the agar overlay medium contained DEAE-Sephadex.

Although homologous interference was readily demonstrable by chick embryo cells incubated with inactivated SP-6 virus particles prior to infection with active SP-6 and LP-7 viruses, homologous interference was not found responsible for the suppressed diameter of the SP-6 plaques.

The rate of adsorption on monolayer cell cultures of chick embryo and rates of inactivation at 37 C in PBS and growth medium were found to be similar for both mutant viruses.

Three separate growth curve experiments were made with chick cells infected with each mutant virus. Infected cells incubated as monolayers with growth medium or agar overlay medium extract or in suspension demonstrated similar results in relation to the lag period of virus release. The LP-7

virus infected cells released newly synthesized virus within 1½ hours, whereas a lag of approximately 3 to 3½ hours was evident for SP-6 infected cells. At 12 hours each SP-6 virus infected cell released approximately 100 plaque forming units (PFU) when incubated as monolayer or cell suspension with growth medium, but when the infected monolayer cells were incubated with agar overlay medium extract only 16 PFU/cell were released. The LP-7 virus infected cells released within 1100 to 2000 PFU/cell when incubated under the conditions described.

Attempts to select mutants from SP-6 and LP-7 viruses which were readily able to infect HeLa, L, FL, mouse embryo, and hamster kidney were not successful.

Each mutant virus was highly lethal for "wet" chicks and suckling mice injected by subcutaneous and intracerebral routes respectively. Fewer than 2 PFU was found sufficient to kill the experimental animals within 5 days.

RESEARCH PROPOSALS

1. The factor in horse serum responsible for suppressing the development of SP-6 and LP-7 plaques should or deserves to be investigated.
2. Growth curve experiments are indicated with WEE virus infected chick embryo cells placed under conditions of homologous interference.
3. Metabolic studies should be carried out with chick embryo cells infected with WEE virus.
4. Efficiency of extracting infectious RNA from poliovirus and WEE virus with phenol should be compared to methods utilizing the standard shaking procedure and sonic-vibration.
5. The correlation between WEE virus particles inactivated by various means and the infectivity of the extracted RNA should be studied.
6. Antiserum will be prepared against primary chick embryo cells in attempts to elucidate whether the peripheral membrane of WEE virus synthesized from such cells were derived from preformed cellular components.
7. It has been reported that chick embryo cells infected with WEE virus and incubated as cell suspensions were unable to synthesize virus in the absence of supernatant protein. The experiments will be repeated to determine the validity

of this report. Homologous interference will be used as a basis for the presence or absence of inactivated WEE virus.

8. WEE mutant virus capable of infecting L cells will be isolated or obtained from elsewhere. Factors responsible for preventing WEE virus from producing plaques on infected L cells will be investigated.
9. The fate of virus particles ingested by phagocytes will be studied.
10. Susceptibility and viremic level of chicks in different age groups injected with a known titer of WEE virus should be compared.